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13. ABSTRACT (Maximum 200 Words)

Our laboratory has recently identified a new protein, Pin1, that is involved in checkpoint control. Pin1 interacts with mitotic phosphoproteins and helps to orchestrate the timing of mitotic events. We found that Pin1 is highly overexpressed in breast cancer. Pin1 levels correlate with the levels of cyclin D1 protein as well as with cyclin D1 mRNA levels in human breast tumors. We have shown that Pin1 is a transcriptional acitvator of cyclin D1. Activation occurrs indirectly through the binding of phosphorylated c-jun. Our data indicate that Pin1 may contribute to neoplastic transformation by causing accumulation of cyclin D1. In addition, Pin1 contributes to cyclin D1 overexpression by regulating the turnover and subcellular localization of beta-catenin and inhibiting its interaction with APC. In Pin1 knock-out mice, mammary epithelial cells fail to undergo the usual proliferative burst during pregnancy, a phenotype that is very similar to the cyclin D1 knock-out. Finally, the PIN1 gene itself is an E2F target gene and essential for Neu/Ras induced transformation of mammary epithelial cells. Crossbreeding experiments have shown that Pin1 knock-out mice are largely protected from breast cancers induced by the Her2/neu or Ras oncogenes.

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Role of the Prolyl Isomerase Pin1 in Oncogenesis

Gerburg M. Wulf

I. Introduction

Phosphorylation of proteins on serine or threonine residues preceding proline (Ser/Thr-Pro) is a major intracellular signaling mechanism. The peptidyl-prolyl cis-trans isomerase Pin1 has recently been shown to isomerize phosphorylated Ser/Thr-Pro motifs in a specific subset of phosphoproteins. This "post-phosphorylation" isomerization leads to conformational changes in the substrate protein and modulates their functional properties. Thus, Pin1 may provide a new post-translational level of control that orchestrates the general increase in serine-phosphorylation and results in an organized set of mitotic events.

Early on Pin1 had been shown to interact with a wide range of mitotic phosphoproteins, and the presence of Pin1 is essential for progression through mitosis. Recently, overexpression of Pin1 was found in a high percentage of human cancers, most exhaustively studied in breast cancer. In cancer cells, Pin1 regulates the expression of cyclin D1 through (a) cooperation with Ras signaling (b) inhibiting the interaction of beta-catenin with APC and (c) direct stabilization of the cyclin D1 protein. In addition, we have recently identified Pin1 itself as a downstream target of oncogenic Ras/Neu signaling via activation of the E2F transcription factor.

II. Body

Task 1 (months 1 – 12)

<u>To examine whether Pin1 or Pin2 are candidate tumor markers for breast cancer</u>

Given that Pin1 regulates the conformation of certain phosphorylated Ser/Thr-Pro motifs, we first asked whether Pin1 expression is aberrantly regulated in human breast cancer. Initially we examined the levels of Pin1 expression in normal and neoplastic breast tissues. In 10 normal and 51 primary human breast cancer tissues examined, we observed striking differences in the levels of Pin1 protein expression. 71 % of grade II tumors and 90 % grade III tumors overexpressed Pin1. Although we observed considerable inter-individual variations, especially in grade II and III tumors, the mean expression level of Pin1 was about 10 times higher in cancer samples than in the normal controls. Pin1 levels positively correlated with the tumor grade in invasive breast cancer, as determined by Kruskal Wallis test. For further details please see reference 1. Given the striking expression data we analyzed whether Pin1 expression correlated with any known breast cancer marker. It turned out that Pin1 levels tightly correlated with cyclin D1 levels. There was a positive correlation with Her2/neu expression that did not reach statistical significance because of the small number of samples. Consistent with the tight correlation between Pin1 and cyclin D1 observed in human breast cancer samples, Pin1 positively regulates cyclin D1 function at the transcriptional level in collaboration with several different oncogenic signaling pathways and also through post-translational stabilizatio (1, 2, 3, 6).

Pin1 binds phosphorylated c-Jun on Ser 63/73-Pro motifs and thereby increases its transcriptional activity towards the cyclin D1 promoter via the AP-1 site. The AP-1 complex regulates a wide range of cellular processes, including cell proliferation, cell death, survival and differentiation. Pin1 binds c-Jun, that is phosphorylated on Ser 63 and 73, and also increases its ability to activate the cyclin D1 promoter in cooperation either with activated JNK or oncogenic Ha-Ras. In contrast, inhibition of endogenous Pin1 reduces the transcriptional activity of phosphorylated c-Jun, indicating that endogenous Pin1 is also required for the optimal activation of c-Jun. Thus, Pin1 is a potent modulator of

phosphorylated c-Jun in inducing cyclin D1 expression, presumably by regulating the conformation of the phosphorylated Ser-Pro motifs in c-Jun. These studies on the biochemical consequences of Pin1 overexpression were performed in addition to what was proposed in Task 1, and this change in strategy was prompted by the clinicophathological data, i.e. the close correlation of Pin1 overexpression with cyclin D1.

The extraction of genomic DNA from the archival tissues for Southern blotting for telomer length or amplification of the *PIN1* gene met with unexpected difficulties: The quality and quantity of the DNA isolated from the tissues that were up to 15 years old was not sufficient for Southern blot analysis. Therefore, these analyses will be performed using a different tissue bank, we will try to do this through the SPORE breast cancer grant sponsored tissue bank at the Harvard Cancer Center.

Pin2 levels in the 50 tumor specimen were significantly lower then in the 10 normal specimen examined, however there was no correlation with tumor grade, LN status of the patient, Cyclin D1 or PCNA or Estrogen Receptor Status. All the pertinent results are presented in the attached paper published in Oncogene (5). The interaction of Pin1 and Pin2 with the tumor suppressor nm23 has not yet been pursued, because we gave the analysis of the oncogenic properties of Pin1 priority. I do want to add, though, that an independent yeast two hybrid screening performed by Dr. Xiao Zhou in our laboratory, has confirmed the interaction of Pin2 with nm23. Work on this task will be started soon.

Task 2 (months 12-36)

To analyze the role that Pin1 and Pin2 play in neoplastic transformation *in vitro* and *in vivo*

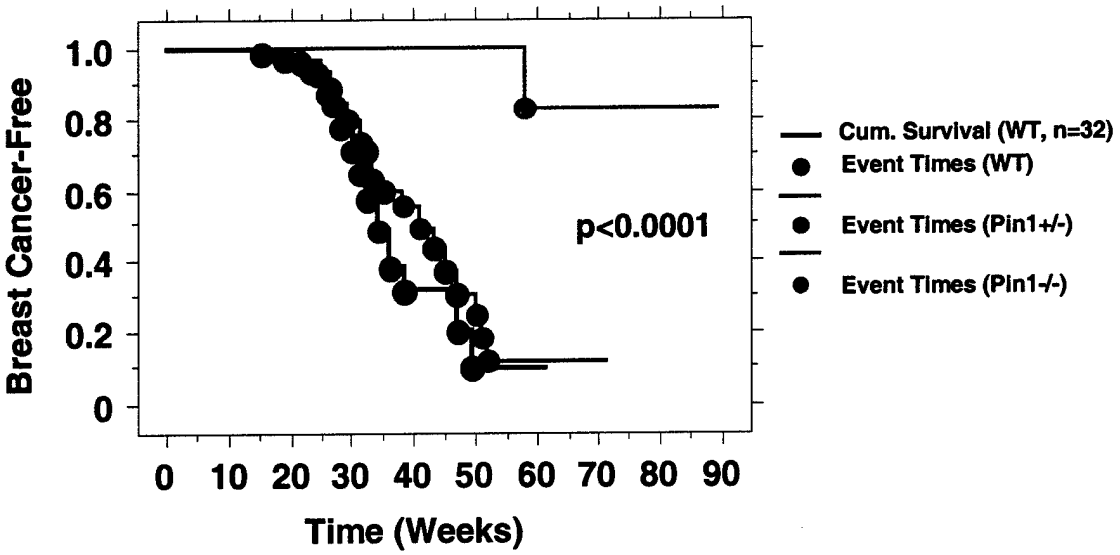
Several MCF7 cell lines inducible for Pin1 had been established. Inducibility had been verified by Western Blotting (see Fig. 3 in reference 1). The induction of Pin1 caused an increase in cyclin D1 expression. However, it turned out that additional (transgenic) Pin1 did not affect the behavior of these cells in the matrigel assay. Also cell cycle ditribution and growth pattern remained unaffected. In essence, it was not possible to alter an already transformed phenotype through an increase in Pin1 levels. Therefore, we resorted to immortalized human mammary epithelial cells that are not yet transformed. We stably transfected GFP-Pin1 and control GFP into MCF-10A cells, a spontaneously immortalized, but non-transformed mammary epithelial cell line that has been widely used for cell transformation studies. Multiple stable cell lines were obtained that had similar properties (3). Consistent with our previous studies (1, 2), cyclin D1 protein levels were elevated in these GFP-Pin1 stable clones as compared with control GFP cells, with cyclin D1 levels correlating with exogenous Pin1 expression levels (Fig. 5A of ref 3). Although there was no detectable difference in cell morphology and growth rate on plastic plates between GFP-Pin1 and control GFP cell lines, overexpression of GFP-Pin1, but not GFP, conferred anchorage-independent cell growth in soft agar (Fig. 5B of ref 3). However, the size and frequency of colonies were much less than those of Neu/Ras-transformed MCF-10A cells (Fig. 5B vs Fig. 6F of ref 3). Moreover, like parental MCF-10A cells, GFP-Pin1 stable cell lines were unable to survive in DMEM media supplemented with10% fetal bovine serum (data not shown), while Neu/Ras-transformed MCF-10A cells can grow normally in this medium (Fig. 6C of ref 3). These data suggest that although overexpression of Pin1 appeared to be insufficient to fully transform MCF-10A cells, it might trigger some early events of cell transformation.

To further investigate this possiblility, we performed a three-dimensional cell differentiation assay using exogenous basement membrane matrix (Matrigel). We found that GFP-expressing cells formed acini with basally polarized nuclear organization, intact cell-cell junctions and visible lumina inside, as indicated by immunostaining with antibodies against the cell-cell junction marker E-cadherin and with the DNA dye TOPRO, followed by confocal microscopy (Fig. 5C of reference 3). Expression of GFP-Pin1 had a profound effect on the morphology and organization of acinar formation. Colonies formed by GFP-Pin1-expressing cells exhibited the disorder in the nuclear polarity and cell arrangement without lumen inside, disruption of basement membrane and impairment in cell-cell junction (Fig.5B lower of reference 3). Furthermore, GFP-Pin1, but not GFP-expressing cells had cell surface spikes protruding into the Matrigel (arrows in Fig. 5D upper of reference 3). These results suggest that Pin1 overexpression can induce events associated with early stages of mammary tumorigenesis.

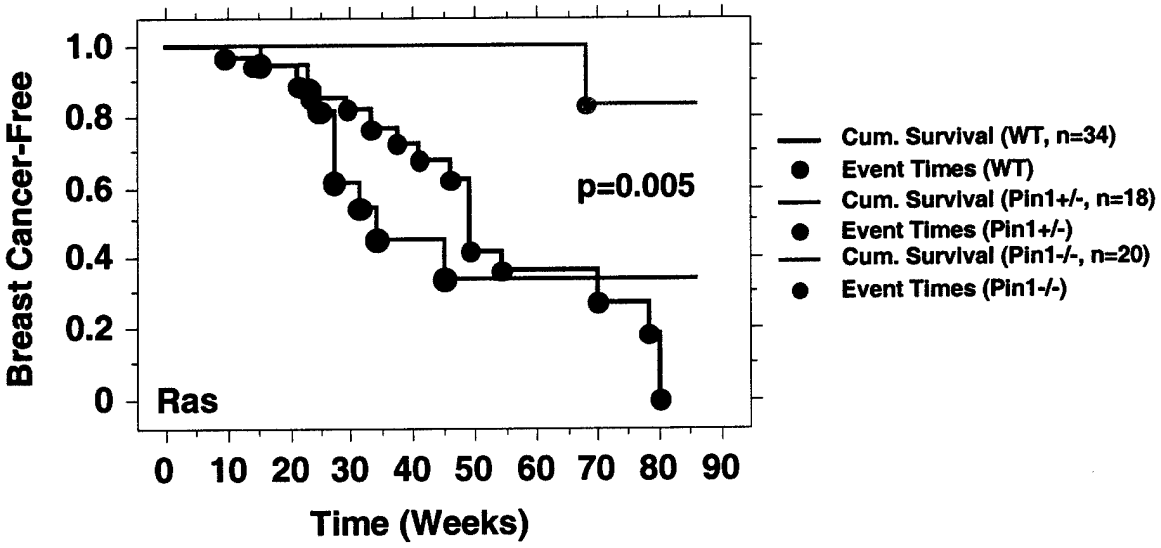
To verify these results in vivo we generated transgenic mice: An expression vector controlling Pin1 expression from the MMTV promoter was constructed, and injected blastocytes in our transgenic facility. We have now a number of transgenic Pin1 mice which we are currently screening for the expression of the Pin1 transgene. Subsequently, I will analyze the incidence of breast cancer in these mice.

In addition, we were able to obtain Pin1 knock-out mice from a Japanese group. I am currently performing breeding experiments with ras and neu transgenic mice. My question here is whether the Pin1 -/- background protects these mice from ras or neu induced breast cancers. Given the ability to control Pin1 levels in these mice, we were interested in determining the effects of Pin1 function on the incidence of mammary carcinomas by monitoring mice that carried one copy of MMTV-c-Neu or -v-Ha-Ras transgene on either WT, $Pin1^{+/-}$ or $Pin1^{-/-}$ background over time. Since the MMTV promoter is induced by pregnancy and also since the breast cancer incidence in male mice is very low, we chose to focus our study on virgin female, as widely used by others. 100% off MMTV-Ras and over 90% of MMTV-Neu transgenic in the wild-type Pin1 background developed one or several breast cancers within 90 weeks of observation (Fig. A, B), which is consistent with the tumor incidence historically observed in these mice However, over 85% of transgenic mice in the $Pin1^{-/-}$ background remained breast cancer-free over the same period of time; only one Neu- or Ras-transgenic mouse in the $Pin1^{-/-}$ background developed breast cancer (Fig. A, B). Kaplan-Meier analysis confirmed a highly significant advantage in disease-free survival for the transgene-positive and $Pin1^{-/-}$ mice as compared to their transgene-positive and $Pin1^{+/+}$ littermates (log rank test, $p<0.0001$ for Neu and $=0.005$ for Ras). Interestingly, there was no survival benefit for the transgene-positive and Pin1 heterozygote mice ($Pin1^{+/-}$) (Fig. A, B). This is consistent with the observation that Pin1 levels in these heterozygote animals were not different from the $Pin1^{+/+}$ mice, indicating that the protective effect is not due to the genetic background of Pin1 knockout mice, but rather specifically due to the loss of Pin1 function. These results indicate that loss of Pin1 function effectively protects against breast cancer induced by oncogenic Neu or Ras.

A. MMTV-Neu



B. MMTV-Ha-Ras



In summary, the analysis of the data obtained in task 1 led to the unexpected discovery that Pin1 activates the transcription of cyclin D1 through binding and activating c-Jun and beta-catenin. Because cyclin D1 plays a much larger role in breast cancer carcinogenesis then nm23, the focus was shifted from studying the interaction on Pin1 with nm23 to its interaction with c-Jun and beta-catenin. The cell line experiments outlined in task 2 were performed but not with MCF-7 as originally planned. Instead non-transformed cells (MCF10A) were used which allowed us to show the transforming properties of Pin1 in cooperation with either Ras or the Her2/neu oncogene. The in vivo data above show that Pin1 knock-out protects mice from Her2/neu or Ras induced breast cancers.

III. Key Research Accomplishments

- Examination of Pin1 levels in 50 primary breast cancer specimen
  Pin1 is overexpressed in 75% of breast cancers
- Correlation of Pin1 levels with clinicopathologic characteristics of the tumors
  Pin1 levels correlate with tumor grade and cyclin D1 levels
- Examination of the Pin1 interactions in vitro
  Pin1 is a transcriptional cotransactivator of the cyclin D1 promoter and interacts with c-jun as well as beta-catenin.
- Pin1's crucial role in Ras and Her2/neu signal transduction cascades were discovered
- Pin1-transgenic mice were generated Pin1 null mice obtained.
- Pin1-knock-out/her2/neu or Ras transgenic mice were generated. It was shown that the Pin1 knock-out phenotype protects mice from oncogene-induced breast cancers.

IV. Reportable Outcomes

- Manuscript5: see attachment, the others were submitted 2002

1. Kishi S, **Wulf G,** Nakamura M and Lu KP. Telomeric Protein Pin2/TRF1 Induces Mitotic Arrest and Apoptosis in Cells with Short Telomeres and is Down-regulated in Human Breast Tumors. **Oncogene** 2001, 20:1497-1508

2. **Wulf G,** Ryo A, Wulf GG, Lee SW, Niu T, Petkova V and Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. **EMBO J.** 2001 20: 3459-3472

3. Ryo A, Nakamura M*, **Wulf G***, Liou Y* and Lu KP. Prolyl isomerase Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. **Nature Cell Biology**, 2001 (3): 793-801
   *These authors contributed equally to this paper

4. Ryo A, Liou Y, **Wulf G**, Nakamura M, Lee S and Lu KP. PIN1 is an E2F Target Gene Essential for Neu/Ras-Induced Transformation of Mammary Epithelial Cells. **MCB** 2002, 22(15): 5281-5295
5. Ablation of the prolyl isomerase Pin1 protects against breast cancer Gerburg Wulf, Priti Garg, Yih-Cherng Liou, Dirk Iglehart* and Kun Ping Lu. Under review at **Cancer Cell**

Patent

Zhou XZ, **Wulf G** and Lu KP. Pin1 as a Marker for Abnormal Cell Growth. U.S. Patent No.60/167,800

V. Conclusions

- The prolylisomerase Pin1 may promote tumor growth through the accumulation of cyclin D1 and through the potentiation Ras and Her2/neu oncogenic signalling. Because of its unique enzymatic function Pin1 could serve as a target for inhibitory drugs.

VI. References

1. **Wulf G,** Ryo A, Wulf GG, Lee SW, Niu T, Petkova V and Lu KP. Pin1 is overexpressed in breast cancer and cooperates with Ras signaling in increasing c-Jun transcriptional activity towards cyclin D1. **EMBO J.** 2001 20: 3459-3472

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4. **Wulf** GM, Liou YC, Ryo A, Lee SW, Lu KP. Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage. **J Biol Chem**. 2002 Oct 17 [epub ahead of print]

5. Kishi S, **Wulf G,** Nakamura M and Lu KP. Telomeric Protein Pin2/TRF1 Induces Mitotic Arrest and Apoptosis in Cells with Short Telomeres and is Down-regulated in Human Breast Tumors. **Oncogene** 2001, 20:1497-1508

6. Liou YC, Ryo A, Huang HK, Lu PJ, Bronson R, Fujimori F, Uchida T, Hunter T, Lu K Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-nullphenotypes. Proc Natl Acad Sci U S A. 2002 Feb 5;99(3):1335-40.

Appendix Cover Sheet

(Revised Manuscript EMBO/2004/47349)

Modeling breast cancer in vivo and ex vivo reveals an essential role of Pin1 in tumorigenesis

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Running title: Essential role of Pin1 in tumorigenesis

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Phosphorylation on certain Ser/Thr-Pro motifs is a major oncogenic mechanism. The conformation and function of phosphorylated Ser/Thr-Pro motifs are further regulated by the prolyl isomerase Pin1. Pin1 is prevalently overexpressed in human cancers and implicated in oncogenesis. However, the role of Pin1 in oncogenesis in vivo is not known. We have shown that Pin1 ablation is highly effective in preventing oncogenic Neu or Ras from inducing cyclin D1 and breast cancer in mice, although it neither affects transgene expression nor mammary gland development. Moreover, we have developed an ex vivo assay to uncover that a significant fraction of primary mammary epithelial cells from Neu or Ras mice display various malignant properties long before they develop tumors in vivo. Importantly, these early transformed properties are effectively suppressed by Pin1 deletion, which can be fully rescued by overexpression of cyclin D1. Thus Pin1 is essential for tumorigenesis and is an attractive anticancer target. The ex vivo assay can be used to study early events of breast cancer development in genetically predisposed mice.

Keywords: Pin1; protein phosphorylation; three dimensional culture; transgenic mice; tumorigenesis

Introduction

Phosphorylation of proteins on certain serine/threonine residues preceding proline (pSer/Thr-Pro) is a major mechanism in regulating cell proliferation and transformation (Blume-Jensen and Hunter, 2001; Hanahan and Weinberg, 2000; Lu, 2004; Lu *et al.*, 2002). Signaling pathways triggered the oncogenes Neu and Ras lead to the activation of various Pro-directed protein kinases, which eventually enhance transcription of the cyclin D1 gene via multiple transcription factors, including E2F, c-jun/AP-1, β-catenin/TCF and NF-κB (Lee *et al.*, 2000). Furthermore, cyclin D1 stability is regulated by post-translational phosphorylation on the Thr286-Pro site by GSK-3β (Alt *et al.*, 2000; Diehl *et al.*, 1998; Diehl *et al.*, 1997). Cyclin D1 plays a pivotal role in the development of breast cancer. Cyclin D1 is overexpressed in 50% of breast cancer patients (Gillett *et al.*, 1994).

Importantly, overexpression of cyclin D1 can transform fibroblasts (Alt *et al.*, 2000; Hinds *et al.*, 1994) and induces mammary tumors (Wang *et al.*, 1994), whereas its inhibition reduces transformed cell growth (Arber *et al.*, 1997). Furthermore, cyclin D1 ablation suppresses the ability of Ha-Ras or c-Neu/HER2/ErbB2 to induce breast cancer in mice (Bowe *et al.*, 2002; Yu *et al.*, 2001). These results indicate that cyclin D1 is an essential downstream target for mammary tumorigenesis induced by oncogenic Neu or Ras, and that a major mechanism in these oncogenic processes is the phosphorylation of Ser/Thr-Pro motifs.

Phosphorylated Ser/Thr-Pro motifs exist in two distinct *cis* and *trans* conformations in native proteins, and their conversion is reduced upon phosphorylation (Lu *et al.*, 1996; Lu *et al.*, 1999; Yaffe *et al.*, 1997). Through isomerization of specific pSer/Thr-Pro bonds, Pin1 induces conformational changes in certain proteins following phosphorylation. These conformational changes have profound effects on catalytic activity, protein dephosphorylation, protein-protein interactions, subcellular location, and/or turnover of many proteins involved in cell signaling and growth regulation (Liou *et al.*, 2002; Ryo *et al.*, 2001; Wulf *et al.*, 2001; Yaffe *et al.*, 1997; Zhou *et al.*, 2000). Thus, Pin1-dependent phosphorylation-specific prolyl isomerization is an important new signaling mechanism (Lu, 2004; Lu *et al.*, 2002).

We have previously found Pin1 overexpression in human breast cancer tissues (Wulf *et al.*, 2001), which has subsequently been confirmed and expanded to a large number of other tumors (Ayala *et al.*, 2003; Bao *et al.*, 2004; Lu, 2003; Ryo *et al.*, 2003a; Ryo *et al.*, 2001). Furthermore, its expression levels closely correlate with cyclin D1 levels in cancer tissues (Ryo *et al.*, 2001; Wulf *et al.*, 2001) and with poor prognosis in prostate cancer (Ayala *et al.*, 2003). Importantly, up-regulation of Pin1 has been shown to elevate cyclin D1 gene expression by activating c-jun/AP-1, β-catenin/TCF and NF-κB transcription factors (Ryo *et al.*, 2001; Ryo *et al.*, 2003b; Wulf *et al.*, 2001). Pin1 can also directly bind to the phosphorylated Thr286-Pro motif in cyclin D1 and stabilize nuclear cyclin D1 protein by inhibiting its export into the cytoplasm (Liou *et al.*, 2002), where it is normally degraded by ubiquitin-mediated proteolysis (Alt *et al.*, 2000; Diehl *et al.*, 1998; Diehl *et al.*, 1997). In addition, the breast epithelial compartment in Pin1-/- adult females failed to

undergo the massive proliferative changes associated with pregnancy (Liou *et al.*, 2002), a phenotype resembling the cyclin D1 null phenotype (Fantl *et al.*, 1995; Sicinski *et al.*, 1995). Finally, Pin1 transcription is enhanced by oncogenic Neu or Ras signaling via E2F activation and it enhances the transformed phenotypes of Neu/Ras-transfected mammary epithelial cells (Ryo *et al.*, 2002). These results from human tissues and cell cultures suggest that Pin1 is a Neu/Ras downstream target that plays an important role in cell transformation and may be an attractive molecular target for cancer diagnosis and therapy (Lu, 2003). However, it has not been shown whether inhibition of Pin1 function affects tumorigenesis, and where it affects the oncogenic process in vivo. This is further complicated by the findings that Pin1 is also important for activation of the tumor suppressor p53 in response to DNA damage in cell cultures (Wulf *et al.*, 2002; Zacchi *et al.*, 2002; Zheng *et al.*, 2002) and for the degradation of endogenous c-Myc (Yeh *et al.*, 2004).

Genetically modified mice by transgenic overexpression and/or gene ablation have long been used to address the role of cancer-related genes in breast cancer in vivo (Bouchard *et al.*, 1989; Bowe *et al.*, 2002; Muller *et al.*, 1988; Sinn *et al.*, 1987; Wang *et al.*, 1994; Yu *et al.*, 2001). For ex vivo model systems, three-dimensional (3D) basement membrane cultures are increasingly used for modeling the biological activities of cancer genes, especially in breast cancer, because they provide a unique opportunity to model the architecture of epithelium in vitro (O'Brien, 2002). Unlike monolayer cultures, mammary epithelial cells (MECs) grown in 3D assay will divide and differentiate in a way that recapitulates glandular development in vivo (Barcellos-Hoff *et al.*, 1989; Debnath *et al.*, 2002; Gudjonsson *et al.*, 2002; Petersen *et al.*, 1992). Furthermore, the introduction of oncogenes into these MECs has been shown to result in a distinct and characteristic phenotype with large and atypically formed structure/colonies with clearly disrupted glandular architecture and filled lumen (Debnath *et al.*, 2002; Muthuswamy *et al.*, 2001; Ryo *et al.*, 2001). Thus, 3D cultures provide the appropriate structural and functional context fundamental for modeling the biological activities of cancer genes.

Here we use a combination of these in vivo and in vitro assays to examine the role of Pin1 in breast cancer. Pin1 ablation in mice efficiently abolishes the ability of MMTV-c-Neu or -v-Ha-

Ras to induce both cyclin D1 and breast cancers, although it has no effect on MMTV-c-Myc. To examine where Pin1 ablation blocks the oncogenic process, we established 3D cultures of primary mammary epithelial derived from these genetically altered mice to study the effects of Pin1 ablation on the growth and differentiation properties of primary MECs directly. In our ex vivo culture system, a significant fraction of primary MECs isolated from Ras or Neu transgenic mice, but not non-transgenic controls, fail to differentiate. These cells instead display various features of malignancy, including forming tumors in nude mice, long before they develop tumors in vivo. Importantly, these early transformed properties are effectively suppressed by Pin1 deletion, which can be fully rescued by overexpression of cyclin D1. These results provide the in vivo and ex vivo evidence for an essential role of Pin1 in early events of tumorigenesis and strongly support Pin1 as an attractive anticancer target. The ex vivo assay can be used to study early events of breast cancer development in genetically predisposed mice.

Results

Pin1 expression in transgenic mice

We have shown that Pin1 is overexpressed in human breast cancer tissues and its expression is increased by activated Neu or Ras (Ryo *et al.*, 2002; Ryo *et al.*, 2001; Wulf *et al.*, 2001). To examine the role of Pin1 in breast cancer induced by Neu and Ras, we crossbred Pin1 knockout (Pin1-/-) mice (Liou *et al.*, 2002) and oncogenic transgenic mice overexpressing an activated rat Neu/Her2/ErbB2 kinase (c-Neu) or v-Ha-Ras under the control of the MMTV promoter (Bouchard *et al.*, 1989; Muller *et al.*, 1988; Sinn *et al.*, 1987). As compared with normal controls, Pin1 levels were consistently increased several-fold in mammary glands or mammary tumors isolated from Neu/Pin1+/+ or Ras/Pin1+/+ animals (Fig. 1A, C). However, no Pin1 protein was detected in mammary gland lysates in all Pin1-/- mice regardless of the transgene (Fig. 1A, C), as expected. Interestingly, we found no significant difference in Pin1 levels between Pin1+/+ and Pin1+/- mice (Fig. 1B), an observation consistent with our previous findings that Pin1 levels are tightly regulated

by multiple mechanisms (Ryo *et al.*, 2002). These results indicate that Pin1 protein is absent in Pin1-/- mice, but remains at wild-type levels in Pin1+/- mice.

Pin1 ablation is highly effective in preventing breast cancers induced by oncogenic Neu or Ras, but not Myc in vivo

We next examined the effects of Pin1 function on the incidence of mammary carcinomas by monitoring virgin mice that carried one copy of MMTV-c-Neu or -v-Ha-Ras transgene on either Pin1+/+, Pin1+/- or Pin-/- background over time. As a control for an oncogene that bypasses cyclin D1 in its mechanism of action, we also crossbred Pin1 knockout mice with MMTV-Myc transgenic mice. As shown for cyclin D1 knockout (Yu *et al.*, 2001), Pin1 knockout did not affect breast cancer induced by MMTV-Myc (Fig. 2C, Table 1). However it had drastic effects on the ability of Ras or Neu to induce breast cancer (Fig. 2 and Table 1). Almost all MMTV-Ras or -Neu transgenic mice developed breast tumors, with the kinetics similar to those historically observed in these mice, which were slightly variable amongst different laboratories likely due to the differences in the genetic background used (Bouchard *et al.*, 1989; Bowe *et al.*, 2002; Muller *et al.*, 1988; Sinn *et al.*, 1987; Yu *et al.*, 2001). However, ~90% of transgenic littermates in the Pin1-/- background remained breast cancer-free over the same period of time (Fig. 2A, B, Table 1). Kaplan-Meier analysis confirmed that Pin1-/- transgenic mice had a highly significant advantage in disease-free survival as compared to Pin1+/+ transgenic littermates (log rank test, $p<0.0001$ for Neu and $=0.0004$ for Ras) (Table 1). Interestingly, there was no survival benefit for Pin1+/- mice over their Pin1-/- littermates (Fig. 2A, B, Table 1). This is consistent with the observation that Pin1 levels in these Pin1+/- animals were not different from the Pin1+/+ mice (Fig. 1B, C), indicating that the protective effect is specifically due to the loss of Pin1 function. As compared with those reported by Yu *et al.* (2001), the overall incidence of salivary gland tumors in our population was much lower precluding statistical analyses, possibly due to different genetic background of our mice. Although further experiments are needed to address the role of Pin1 on other cancers, these results indicate

that loss of Pin1 function is highly effective in preventing breast cancer induced by oncogenic Neu or Ras.

Pin1 ablation affects neither the development of virgin mammary glands nor the expression of the transgenes

Given this striking protection of Pin1 ablation against breast cancer, we were interested in determining the effects of Pin1 ablation on oncogenic processes. It has been reported that mammary glands in Pin1-/- or MMTV-Neu or -Ras transgenic virgin females develop normally (Liou *et al.*, 2002; Yu *et al.*, 2001), although Pin1-/- mammary glands fail to undergo the massive proliferation during pregnancy (Liou *et al.*, 2002). To address the question whether the combination of the transgene with Pin1 deletion affected mammary gland development, we performed whole mount and histological analyses (Liou *et al.*, 2002; Yu *et al.*, 2001). Morphometric analysis of carmine-stained whole-mounts of the virgin mammary glands revealed inter-individual variations, but no significant difference in the number of primary ducts, secondary branches or end buds between Pin1+/+ and Pin1-/- mice carrying the Ras or Neu transgene (Table 2, Fig. S1A). All virgin female mice developed proper mammary ducts with an intact lumen and again there was no detectable difference between Pin1+/+ and Pin1-/- background (Fig. S1B).

We next asked whether Pin1 ablation could affect the expression of the c-Neu or Ha-Ras transgene. It has been shown that expression levels of these transgenes are typically low in non-neoplastic mammary glands, although they tend to be much higher in mammary tumors (Muller *et al.*, 1988, Bouchard, 1989 #2245; Sinn *et al.*, 1987; Yu *et al.*, 2001). In addition, the transgenes are only expressed in MECs, not in the surrounding architectural and fat pad tissue, which make up for the bulk of the mammary gland in the virgin mouse (Fig. 1E). Therefore, we used immunohistochemistry as well as immunoblotting analyses to detect the expression of the c-Neu or Ha-Ras transgene. Both assays showed no detectable difference in transgene expression in mammary glands between Pin1+/+ and Pin1-/- mice (Fig. 1E, F). These results indicate that Pin1 ablation does not affect the expression of the transgenes.

Pin1 ablation effectively blocks the induction of cyclin D1 by Neu or Ras

It has been shown that in Neu- or Ras-transgenic mice, cyclin D1 is induced, which is essential for Neu or Ras-induced breast cancer (Yu *et al.*, 2001). We had previously shown that Pin1 positively regulates cyclin D1 levels by transcriptional activation and post-translation stabilization in response to growth signals in vitro (Liou *et al.*, 2002; Ryo *et al.*, 2001; Wulf *et al.*, 2001). These results suggest that loss of Pin1 might block the induction of cyclin D1 in Neu- or Ras-transgenic mice. Therefore, we analyzed cyclin D1 expression in mammary glands derived from different genetically modified mice by immunoprecipitation, followed by immunoblotting analysis with anti-cyclin D1 antibodies. As shown (Liou *et al.*, 2002; Yu *et al.*, 2001), cyclin D1 was lower in Pin1-/- mice, but induced in Neu or Ras transgenic mice in the Pin1+/+ genetic background (Fig. 3A). However, in the Pin1-/- genetic background, cyclin D1 was barely induced in Neu or Ras transgenic mice (Fig. 3A). To confirm these results, we performed immunohistochemistry using anti-cyclin D1 antibodies. While cyclin D1 immunostaining signals were readily detected in MECs in Neu/Pin1+/+ or Ras/Pin1+/+, there were barely any cyclin D1 signals in Neu/Pin1-/- or Ras/Pin1-/- mice (Fig. 3B). These results indicate that Pin1 ablation effectively blocks the induction of cyclin D1 by Neu or Ras.

Pin1 ablation does not affect the differentiation of primary mouse mammary epithelial cells (MECs) in 3 dimensional (3D) cultures

Given that Pin1 ablation is effective in suppressing breast cancer induced by Neu or Ras, we next established ex vivo cultures of primary MECs derived from these mice to determine whether Pin1 deletion affects the growth and differentiation properties of mammary epithelial cells (MECs). Our hypothesis was that these ex vivo cultures might allow us to discern growth patterns between MECs that were isolated from Ras or Neu transgenic animals and "programmed" to develop into breast

cancer from those that were isolated from non-transgenic mice or transgenic mice in Pin1-/- background and "programmed" not to develop into breast cancer.

Primary MECs were isolated from morphologically normal mammary glands of wild-type mice or Neu or Ras transgenic mice in Pin1+/+ or Pin1-/- background at ages of 3-4 months. To examine the possibility that small microscopic foci of tumors that were macroscopically not yet detectable might affect the ex vivo culture, we performed histological examinations of the inguinal mammary gland that was contralateral to the mammary gland used for ex vivo cultures and did not find any invasive or in situ carcinoma at these early stages. Furthermore, we did not find any significant difference among these different genetic backgrounds when primary MECs were cultured on collagen-coated dishes (2D cultures) (Fig. S2). All cells appeared as a rather homogenous population that grew in an anchorage-dependent fashion, required growth factor for survival, and eventually stopped growing within 2 weeks ex vivo. We then plated primary MECs as single cell suspension in reconstituted basement membrane using modified culture media (3D cultures), as described in Materials and Methods. MECs from Pin1+/+ or Pin1-/- mice began to form globular colonies, and the cells in the center started to undergo apoptosis. These globular colonies then developed into organized and polarized acinus-like colonies with an intact lumen by day 10, followed by a stop in cell growth by day 20 of cultures (Fig. 4A, data not shown). These orderly differentiated "Regular" colonies exhibited polarized expression of E-cadherin (Fig. 4A) and showed lost or low-level Ki67 expression (Fig. 5E). These in vitro differentiation patterns are similar to those described of human primary MECs and normal MEC cell line MCF10A (Debnath *et al.*, 2002; Gudjonsson *et al.*, 2002). They indicate that the deletion of Pin1 does not affect orderly and terminal differentiation of primary MECs ex vivo.

Primary MECs of Neu or Ras mice display various malignant properties, including forming tumors in nude mice, long before they develop tumors in vivo

We found distinct and strikingly different differentiation patterns for MECs derived from Neu or Ras transgenic as opposed to wild-type mice (Fig.4, 5, S4), although there were considerable inter-

individual variations (Fig. 6A-D). Neu and Ras MECs tended to have an overall higher plating efficiency and higher colony counts than non-transgenic cells (Fig. 6A), suggesting that Ras and Neu transgenic animals may have an expanded MEC progenitor cell pool. The majority of primary MECs differentiate into well-differentiated round acinar colonies (Fig. 5 and 6B), as is the case for almost all cells derived from wild-type mice (Fig. 5A, F, first panel, 6B "Regular"). However, we observed the stochastic, independent emergence of large, multi-acinar colonies with lumen filled, which were rarely observed in non-transgenic MECs (Fig. 5A, F, second panel, 6C "Irregular"). More interestingly, we also observed expansive colonies with invading cells emerging from the original acinar colonies (Fig. 5A, F, third panel, 6D). These "Cancer-like" colonies were reproducibly observed in all primary MEC cultures derived from Neu or Ras transgenic mice, but not from any non-transgenic mice (Fig. 5). H&E staining showed that the "Regular" colonies were formed by uniform MECs with basally polarized nuclear organization, small nuclei and abundant cytoplasm (Fig. 5B, G). "Irregular" colonies were large, often had multiple acini, and their lumia were characteristically filled (Fig. 5B, G). "Cancer-like" colonies had disrupted cell polarity, cell surface spikes invading into the Matrigel, persistent mitotic figures, large and irregular nuclei, and high nuclear/cytoplasmic ratio (Fig. 5B, G).

Loss of E-cadherin expression, breaching of the basement membrane and continuous cell proliferation are some features of breast cancer cells (D'Ardenne *et al.*, 1991; Moll *et al.*, 1993; Pavelic *et al.*, 1992). Therefore, we next performed immunofluorescence staining in situ on these colonies with antibodies against E-cadherin, α6 integrins and Ki67. Consistent with the histological features, we found orderly and mostly basal expression of E-cadherin in the "Regular" colonies (Fig. 5C, H). E-cadherin expression was lost in those cells that filled the lumen in "Irregular" colonies and even more obviously in "Cancer-like" colonies (Fig. 5C, H). Furthermore, "Regular" acini had the orderly, basal α6 integrin expression encircling the acini fully (Fig. 5D), a characteristic of normal mammary epithelial acini (D'Ardenne *et al.*, 1991). This was in sharp contrast to disorganized α6 integrin expression in "Cancer-like" acini, where basal α6 integrin expression pattern was completely disrupted and epithelial cells broke through and invaded into the

basal membrane-containing Matrigel (Fig. 5D). Moreover, "Irregular" and "Cancer-like" colonies continued to express Ki67 beyond day 20 of culture, while "Regular" acini tended to be Ki67-negative (Fig. 5E). These results together indicate that a significant fraction of primary Neu or Ras MECs fail to differentiate, but continuously grow into invasive colonies. Interestingly, these abnormal properties resemble to those of the normal MECs MCF10A transformed with oncogenes in vitro (Debnath *et al.*, 2002; Muthuswamy *et al.*, 2001).

To further confirm that the "Regular" colonies are mostly composed of non-dividing terminally differentiated cells and the "Irregular" colonies contain actively dividing cells, we picked "Regular" and "Irregular" colonies separately at day 21and assayed for secondary colony formation. Although cells derived from "Regular" colonies gave rise to only very few "Regular" secondary acinar colonies, "Irregular" colonies gave rise to multiple "Irregular" colonies. These results indicate that the cells in these "Irregular" colonies retain their proliferative capacity, and that these colonies are indeed the result of clonal expansion of a distinct type of MECs (Fig. 6E).

Finally, to confirm that these "Cancer-like" colonies indeed contain cancer cells, we surgically transplanted these colonies into nude mice to examine their ability to form tumors. 1-2 months later after the transplantation, tumors were visually identified at 50% of sites that were transplanted with "Cancer-like" colonies formed by MECs derived from Neu transgenic mice, but not from control MECs (Fig. 6F, data not shown). Taken together, these results indicate that a significant fraction of primary MECs derived from morphologically and histologically normal mammary gland of Neu or Ras mice exhibit the malignant phenotype ex vivo.

Ablation of Pin1 suppresses early transformed properties of Neu or Ras MECs

Given that primary MECs derived from Neu- or Ras-transgenic mice display the transformed phenotype ex vivo long before they produce tumors in vivo, we then asked whether this transformed phenotype is affected by Pin1 ablation. Like wild-type cells, Neu/Pin1-/- MECs and Ras/Pin1-/- MECs tended to have lower colony counts than their Pin1+/+ counterparts (Fig. 6A), indicating that loss of Pin1 function may prevent the increase in the MEC progenitor cells seen in Neu or Ras

transgenic mice. Importantly, the frequency of "Irregular" colonies was greatly reduced in Neu/Pin1-/- or Ras/Pin1-/- MECs, as compared those from Neu/Pin1+/+ or Ras/Pin1+/+ cells (Fig. 5, 6C). Furthermore, "Cancer-like" colonies were absent from Neu/Pin1-/- derived cultures and very rare in Ras/Pin1-/- cultures (Fig. 5, 6D). Moreover, colonies derived from Neu/Pin1-/- MECs failed to form any tumors when transplanted into nude mice (Fig. 6F). These data indicate that Pin1 ablation effectively suppresses the early transformed phenotype of Ras or Neu MECs ex vivo.

Overexpression of cyclin D1 in Neu/Pin1-/- primary MECs rescues their malignant phenotype

The above results indicate that the Pin1-/- genetic background, Neu or Ras fails to transform MEC and to induce breast cancer as well as to increase cyclin D1 expression. Since cyclin D1 is essential for Neu or Ras to induce breast cancer (Bowe *et al.*, 2002; Yu *et al.*, 2001), we asked whether the failure of Neu or Ras to induce cell transformation and breast cancer in the Pin1-/- genetic background is due to the absence of cyclin D1 induction. To address this question, we used retroviral gene transfer with concomitant GFP expression to deliver cyclin D1 or its T286A mutant to primary MECs derived from Neu/Pin1+/+ mice, as described (Debnath *et al.*, 2002). Based on GFP expression, the infection efficiency was over 80% and transgene expression was confirmed by immunoblot (Fig. S4). Importantly, when infected with cyclin D1 or but not the control vector, Neu/Pin1-/- MECs generated "Cancer-like" colonies (Fig. 7A), with a similar incidence than Neu/Pin1+/+ cells (Fig. 7C, 6D). These "Cancer-like" phenotype was even more obvious when infected with the cyclin $D1^{T286A}$ mutant (Fig. 7B, C), a mutant known to be more stable and potent in transforming cells (Alt *et al.*, 2000). These results further support the idea that the inhibition of tumorigenesis by Pin1 ablation is due to the suppression of cyclin D1.

Discussion

We have shown that Pin1 ablation is highly effective in preventing oncogenic Neu or Ras from inducing cyclin D1 and breast cancer in mice, although it neither affects transgene expression nor mammary gland development. Moreover, we have developed an ex vivo assay to uncover that a fraction of non-neoplastic primary MECs derived from Ras or Neu mice display the transformed phenotype long before they develop breast cancer in vivo. These cells fail to differentiate normally, but continuously grow into large, irregular and invasive colonies that display various features of cancer cells, including forming tumors in nude mice. Importantly, this early transformed phenotype is not found in wild-type MECs and effectively suppressed by Pin1 ablation, which can be fully rescued by cyclin D1 overexpression. Therefore, the protective effect of Pin1 ablation is inherent to the MECs, which can undergo normal differentiation but are resistant to oncogenic transformation. This study provides the in vivo and ex vivo evidence for an essential role of Pin1 in early events of tumorigenesis and supports Pin1 as an attractive anticancer target. The ex vivo assay can be used to study early events of breast cancer in genetically predisposed mice.

Our data demonstrate an essential role of Pin1 for tumorigenesis induced by Neu and Ras in vivo. The observations that Pin1 ablation does not affect transgene expression or mammary gland development suggest that it likely affects signaling pathways that are activated by oncogenic Ras or Neu. Indeed, Pin1 deletion suppresses the induction of cyclin D1 by oncogenic Ras or Neu, which are consistent with various previous in vitro studies. Pin1 expression is upregulated by c-Neu or Ha-Ras via E2F (Ryo *et al.*, 2002). Pin1 upregulation in turn enhances signaling downstream from Neu and Ras and also stabilizes cyclin D1 (Liou *et al.*, 2002; Ryo *et al.*, 2001; Ryo *et al.*, 2003b; Wulf *et al.*, 2001). These critical roles of Pin1 in transcriptional and post-translation regulation of cyclin D1 may explain why Pin1 deletion blocks the induction of cyclin D1 by Neu or Ras. The lack of cyclin D1 induction in Pin1 null mice is highly significant given that cyclin D1 is an essential downstream target for mammary tumorigenesis. Our findings that overexpression of cyclin D1 or its constitutively active mutant fully rescues the malignant phenotype of Neu/Pin1-/-

MECs further underscores the importance of cyclin D1 in Neu-induced breast cancer. Furthermore, this is also consistent with the recently reported protective effect of the cyclin D1 ablation on breast cancers induced by Ha-Ras or c-Neu, but not Myc (Bowe *et al.*, 2002; Yu *et al.*, 2001). Therefore, one molecular mechanism by which Pin1 ablation protects against breast cancer is the suppression of cyclin D1 induced by Ras or Neu.

To further determine how Pin1 ablation protects against breast cancer, we have established a colony formation assay of non-neoplastic primary MECs based on 3D cultures. Colony formation assays on semi-solid media have been used extensively in the study of hematopoietic progenitor cells (Lowenberg *et al.*, 1993; Senn *et al.*, 1967). The underlying principle is that single stem and progenitor cells can give rise to aggregates of terminally differentiated cells. These assays not only allow the distinction of different types of hematopoietic progenitor cells, but also the distinction between malignant cells (Lowenberg *et al.*, 1993; Senn *et al.*, 1967). In our approach, we have employed this concept to the study of primary mammary epithelial cells derived from mice carrying the Neu or Ras transgene. It is based on the emerging evidence that many, if not all, breast cancers may be derived from a pool of mammary epithelial cell progenitor cells (Smalley and Ashworth, 2003). We hypothesized that a colony formation assay, in which only mammary epithelial cells that can divide give rise to colonies, might be a tool to distinguish MECs that have the potential to become malignant from those that differentiate normally.

Indeed, the 3D colony cultures can uncover that the growth properties of non-neoplastic primary MECs from Ras/Pin1+/+ or Neu/Pin1+/+ mice differed greatly from those from Ras/Pin1-/- or Neu/Pin1-/- mice. Mouse primary MECs from Pin1+/+ or Pin1-/- mice can differentiate into well organized round acinar colonies, like primary human MECs and MCF10A (Debnath *et al.*, 2002; Gudjonsson *et al.*, 2002). However, primary MECs derived from Neu/Pin1+/+ or Ras/Pin1+/+ mice revealed a surprising pleomorphism in the 3D cultures. A significant fraction of these colonies derived from morphologically normal MECs ex vivo generated "Irregular" colonies that resemble those generated by mammary epithelial cell lines MCF10A transformed with oncogenes (Debnath *et al.*, 2002; Muthuswamy *et al.*, 2001). These colonies bear

features of malignancy in that they have filled lumina with epithelial cells that lose E-cadherin expression, retain Ki67 expression and continue to grow into large and bizarre bodies. Furthermore, there are rare "Cancer-like" colonies that emerge, with loss of basement membranes and cell-cell junction, and invasion into the reconstituted basement membrane, persistent mitotic figures, large and irregular nuclei, and high nuclear/cytoplasmic ratio. Consistent with the emergence of tumors in vivo, these "Cancer-like" colonies emerge in a stochastic fashion. The low frequency of these ex vivo "Cancer-like" colonies is not surprising, given that the frequency of "in vivo" cancers is even 2 or more decimals lower. Significantly, the deletion of Pin1 effectively suppresses the ability of c-Neu and Ha-Ras to induce these "Cancer-like" colonies in vitro, which correlates with its ability to prevent Ras or Neu-induced breast cancer in vivo. Therefore, the protection of breast cancer by Pin1 deletion is inherent to MECs.

The ex vivo 3D assay of primary mouse MECs has several intriguing features, especially given availability of a large number of well-established cancer mouse models. As a colony formation assay it is based on the clonal expansion of single MECs, and therefore assays only those primary cells that have retained the capacity for proliferation. Therefore, this culture system may allow us to identify MECs that have already undergone the "programming" towards malignant transformation, but do not yet exhibit the malignant phenotype in vivo or in a 2D culture system. Furthermore, this culture system may afford to study the very early genetic events that precede phenotypic change and in vivo tumor formation. In addition, the "ex vivo" tumorigenesis may allow the investigator to study growth patterns of disorganized, hyperplastic and invasive growth of primary MECs in the absence of other structural cell types of the organ and in the absence of other growth modulating influences, that are usually present in the organism. Because of its simplicity, it is a very controllable setting that may allow studying the contribution of the individual cellular and humoral components to the oncogenic process.

Although further studies are required to discern the downstream pathways and genes through which Pin1 regulates tumorigenesis, our study reveals an in vivo function for Pin1 as an essential component of the tumorigenesis pathway initiated by oncogenic activated Neu or Ras.

Pin1 may therefore represent an attractive target for developing anticancer agents. Several other factors also support targeting Pin1 in cancer therapy. Pin1 is an enzyme with an extraordinarily high substrate specificity and well-defined active site. Furthermore, Pin1 is broadly overexpressed not only in breast cancer, but also in a number of other cancers such as prostate, lung and colon cancers (Wulf *et al.*, 2001). In prostate cancer, Pin1 overexpression correlates with poor prognosis (Ayala *et al.*, 2003). Given that Pin1 functions as a catalyst for many known oncogenic pathways rather than an oncogene itself (Lu, 2003), one can envision that incorporating a Pin1 inhibitor in classical or targeted anticancer treatment regimen may greatly enhance the efficacy of these agents.

Experimental Procedures

Animals

MMTV-v-Ha-Ras, MMTV-c-myc (Sinn *et al.*, 1987) or MMTV-c-Neu (Bouchard *et al.*, 1989; Muller *et al.*, 1988) transgenic mice in FVB genetic background were purchased from Charles River Laboratories. Transgenic animals were bred with Pin1-/- mice, which are in mixed genetic background of 129:C57BL6, as described (Liou *et al.*, 2002). Transgenic heterozygous animals were then bred with heterozygous females to obtain the experimental cohort that was followed for the development of tumors. Only virgin females were enrolled in the study and they were examined for the development of tumors twice weekly. For histological sections, the glands were fixed in Bouin's solution, and standard histology sections were stained with Hematoxylin/Eosin. The slides were reviewed with a rodent histopathologist. For whole mount preparations, an inguinal gland was removed and stained with carmine red as described (Liou *et al.*, 2002). Primary ducts, side branches and end buds were counted under a dissecting microscope. Immunohistochemistry to detect cyclin D1, Ha-Ras and c-Neu was done as described (Liou *et al.*, 2002).

Immunoblotting and immunohistochemistry

Immunoblotting and immunohistochemistry were performed as described (Wulf *et al.*, 2001). Briefly, tissue lysates from inguinal mammary glands were prepared and spun, followed by incubation for 10 min at 4°C to allow for solidification of the fat component. The lower, liquid phase was aspirated. Immunoprecipitation experiments were done using antibody-coupled agarose beads for the c-Neu antigen (sc-7301 AC) and the H-ras antigen (sc-35 AC), while immunoblotting was done with antibodies sc-520 for H-Ras, and anti c-Neu Ab-3 from Oncogene. Polyclonal antibody sc 718 was used for immunoprecipitation and immunoblotting of cyclin D1 (sc 718), all antibodies except for anti c-Neu were purchased from Santa Cruz Biotechnology. For immunohistochemistry, both tissue sections and matrigel-embedded cultures were fixed with Bouin's solution and paraffin-embedded. The sections were deparaffinized, rehydrated and subjected to antigen retrieval by boiling them for 10 min in 1x Antigen retrieval solution (Vectra). Slides were blocked with PBS/5%

goat serum, and then incubated with antibodies against Ha-Ras, cyclin D1 and c-Neu. They were then processed with biotinylated secondary antibody, and developed using the Vectorstain kit and DAB solution (Vector Labs).

Culture of primary mouse MECs ex vivo

Primary MECs were isolated from the morphologically and histologically normal mammary glands from virgin mice ages 3-4 months. The mammary glands were mechanically disaggregated, and then subjected to collagenase digestion (100 mg/ml) at 37°C with gentle shaking (100 rpm) in a total volume of 10 ml DMEM/F12 per mammary gland for 2 hours. The digested material was then washed with HBSS/2% horse serum (Gibco) 3 times, followed by 1 wash with HBSS. The pellet was resuspended in trypsin and digested for another 10 min at 37°C, followed by neutralization with 10% horse serum, and a final wash with HBSS. The pellet was resuspended in MEGM and plated on 6 cm culture dishes that had been coated with collagen (50 mcg/ml). After 3-5 days in culture the mammary epithelial cells were trypsinized, washed with HBSS/10% horse serum, counted and resuspended in DMEM/F12 supplemented with Insulin 5 ng/ml, Choleratoxin 100 ng/ml, Hydrocortisone 500 ng/ml at 100,000 cells/ ml. The suspension was then diluted 1:1 with MEGM/4% Matrigel (BD Biosciences 354230) and plated in Falcon Culture slides (BD 354118) that had been coated with Matrigel, at 10,000 cells per chamber. For immunofluorescence, the colonies in Matrigel were fixed with 2% freshly prepared paraformadehyde and analyzed using a BioRad confocal microscope, as described (Debnath *et al.*, 2002; Ryo *et al.*, 2002). For histology, the fixed colonies were paraffin-embedded and processed like tissue blocks. Antibodies used were anti-Ecadherin (Becton), Rat anti Ki67 (Dako) and Rat anti alpha 6 integrin (G0H3, Chemicon).

Retroviral Gene Transfer

Cyclin D1 and cyclin D1 286A in pBabe were a gift from Drs. J. Debnath and J. Brugge. Murine cyclin D1 and its constitutively active mutant cyclin D1 286A were subcloned into the retroviral vector WIRES from Dr. A. M. Kenney, in which Blasticidin resistence sequence had been replaced

with GFP. The constructs were co-transfected with VSV and gag-pol into the packaging cell line 293 EBNA as described Debnath, 2002 #2184]. The primary MECs were infected on three consecutive days for 6 hours each. On day 4 they were subjected to 3D culture assay.

Tumorigenicity Assay

100,000 primary MECs isolated from Neu Pin1+/+ or Neu/Pin-/- mice were subjected to 3D cultures for 21 days. All developing structures were harvested and resuspended in 100 μl MEGM/4% Matrigel. They were injected subcutaneously under the back skin of 5- to 6-week-old NCr athymic female nude mice (Taconic), in duplicates each (right and left flank). Mice were observed weekly for the visual appearance of tumors at injection sites.

Statistical analysis

Nine cohorts were considered for the analysis of the endpoint, disease-free survival. The Kaplan-Meier method was used to estimate disease-free survival for each cohort. The significance of the differences in disease-free survival among the cohorts was determined with the use of log-rank (Mantel-Cox) test.

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References

Alt, JR, Cleveland, JL, Hannink, M and Diehl, JA (2000) Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes Dev* **14**: 3102-3114.

Arber, N, Doki, Y, Han, EK, Sgambato, A, Zhou, P, Kim, NH, Delohery, T, Klein, MG, Holt, PR and Weinstein, IB (1997) Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. *Cancer Res* **57**: 1569-1574

Ayala, G, Wang, D, Wulf, G, Frolov, A, Le, R, Wheeler, T, Sowadski, JM, Lu, KP and Bao, L (2003) Pin1 is a novel prognostic marker in prostate cancer. *Cancer Research* **63**: 6244-6251

Bao, L, Sauter, G, Sowadski, J, Lu, KP and Wang, D (2004) Prevalent overexpression of prolyl isomerase Pin1 in human cancers. *Am. J. Pathol* **164**: 1727-1737

Barcellos-Hoff, MH, Aggeler, J, Ram, TG and Bissell, MJ (1989) Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* **105**: 223-235.

Blume-Jensen, P and Hunter, T (2001) Oncogenic kinase signalling. *Nature* **411**: 355-365

Bouchard, L, Lamarre, L, Tremblay, PJ and Jolicoeur, P (1989) Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. *Cell* **57**: 931-936.

Bowe, DB, Kenney, NJ, Adereth, Y and Maroulakou, IG (2002) Suppression of Neu-induced mammary tumor growth in cyclin D1 deficient mice is compensated for by cyclin E. *Oncogene* **21**: 291-298.

D'Ardenne, AJ, Richman, PI, Horton, MA, McAulay, AE and Jordan, S (1991) Co-ordinate expression of the alpha-6 integrin laminin receptor sub-unit and laminin in breast cancer. *J Pathol* **165**: 213-220.

Debnath, J, Mills, KR, Collins, NL, Reginato, MJ, Muthuswamy, SK and Brugge, JS (2002) The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. *Cell* **111**: 29-40.

Diehl, JA, Cheng, M, Roussel, MF and Sherr, CJ (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* **12**: 3499-3511.

Diehl, JA, Zindy, F and Sherr, CJ (1997) Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev* **11**: 957-972.

Fantl, V, Stamp, G, Andrews, A, Rosewell, I and Dickson, C (1995) Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev* **9**: 2364-2372

Gillett, C, Fantl, V, Smith, R, Fisher, C, Bartek, J, Dickson, C, Barnes, D and Peters, G (1994) Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. *Cancer Res* **54**: 1812-1817

Gudjonsson, T, Ronnov-Jessen, L, Villadsen, R, Rank, F, Bissell, MJ and Petersen, OW (2002) Normal and tumor-derived myoepithelial cells differ in their ability to interact with luminal breast epithelial cells for polarity and basement membrane deposition. *J Cell Sci* **115**: 39-50.

Hanahan, D and Weinberg, RA (2000) The hallmarks of cancer. *Cell* **100**: 57-70

Hinds, PW, Dowdy, SF, Eaton, EN, Arnold, A and Weinberg, RA (1994) Function of a human cyclin gene as an oncogene. *Proc Natl Acad Sci U S A* **91**: 709-713

Lee, RJ, Albanese, C, Fu, M, D'Amico, M, Lin, B, Watanabe, G, Haines, GK, 3rd, Siegel, PM, Hung, MC, Yarden, Y, Horowitz, JM, Muller, WJ and Pestell, RG (2000) Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol Cell Biol* **20**: 672-683

Liou, YC, Ryo, R, Huang, HK, Lu, PJ, Bronson, R, Fujimori, F, Uchidafl, U, Hunter, T and Lu, KP (2002) Loss of Pin1 function in the mouse causes phenotypes resembling cyclin D1-null phenotypes. *Proc. Natl. Acad. Sci. USA* **99**: 1335-1340

Lowenberg, B, van Putten, WL, Touw, IP, Delwel, R and Santini, V (1993) Autonomous proliferation of leukemic cells in vitro as a determinant of prognosis in adult acute myeloid leukemia. *N Engl J Med* **328**: 614-619.

Lu, KP (2003) Prolyl isomerase Pin1 as a molecular target for cancer diagnostics and therapeutics. *Cancer Cell* **4**: 175-180

Lu, KP (2004) Pinning down cell signaling, cancer and Alzheimer's disease. *TiBS* **29**: 200-209

Lu, KP, Hanes, SD and Hunter, T (1996) A human peptidyl-prolyl isomerase essential for regulation of mitosis. *Nature* **380**: 544-547

Lu, KP, Liou, YC and Zhou, XZ (2002) Pinning down the proline-directed phosphorylation signaling. *Trends Cell Biol* **12**: 164-172

Lu, PJ, Zhou, XZ, Shen, M and Lu, KP (1999) A function of WW domains as phosphoserine- or phosphothreonine-binding modules. *Science 283*: 1325-1328

Moll, R, Mitze, M, Frixen, UH and Birchmeier, W (1993) Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *Am J Pathol* **143**: 1731-1742.

Muller, WJ, Sinn, E, Pattengale, PK, Wallace, R and Leder, P (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* **54**: 105-115.

Muthuswamy, SK, Li, D, Lelievre, S, Bissell, MJ and Brugge, JS (2001) ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol* **3**: 785-792.

O'Brien, LE (2002) Opinion: Building epithelial architecture: insights from three-dimensional culture models. *Nat Rev Mol Cell Biol* **3**: 531-537.

Pavelic, ZP, Pavelic, L, Lower, EE, Gapany, M, Gapany, S, Barker, EA and Preisler, HD (1992) c-myc, c-erbB-2, and Ki-67 expression in normal breast tissue and in invasive and noninvasive breast carcinoma. *Cancer Res* **52**: 2597-2602.

Petersen, OW, Ronnov-Jessen, L, Howlett, AR and Bissell, MJ (1992) Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. *Proc Natl Acad Sci U S A* **89**: 9064-9068.

Ryo, A, Liou, YC, Lu, KP and Wulf, G (2003a) Prolyl isomerase Pin1: a catalyst for oncogenesis and a potential therapeutic target in cancer. *J Cell Sci* **116**: 773-783

Ryo, A, Liou, YC, Wulf, G, Nakamura, N, Lee, SW and Lu, KP (2002) Pin1 is an E2F target gene essential for the Neu/Ras-induced transformation of mammary epithelial cells. *Mol Cell Biol* **22**: 5281-5295

Ryo, A, Nakamura, N, Wulf, G, Liou, YC and Lu, KP (2001) Pin1 regulates turnover and subcellular localization of beta-catenin by inhibiting its interaction with APC. *Nature Cell Biol* **3**: 793-801

Ryo, A, Suizu, F, Yoshida, Y, Perrem, K, Liou, YC, Wulf, G, Rottapel, R, Yamaoka, S and Lu, KP (2003b) Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol Cell* **12**: 1413-1426

Senn, JS, McCulloch, EA and Till, JE (1967) Comparison of colony-forming ability of normal and leukaemic human marrow in cell culture. *Lancet* **2**: 597-598.

Sicinski, P, Donaher, JL, Parker, SB, Li, T, Fazeli, A, Gardner, H, Haslam, SZ, Bronson, RT, Elledge, SJ and Weinberg, RA (1995) Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* **82**: 621-630

Sinn, E, Muller, W, Pattengale, P, Tepler, I, Wallace, R and Leder, P (1987) Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell* **49**: 465-475.

Smalley, M and Ashworth, A (2003) Stem cells and breast cancer: A field in transit. *Nat Rev Cancer* **3**: 832-844.

Wang, TC, Cardiff, RD, Zukerberg, L, Lees, E, Arnold, A and Schmidt, EV (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* **369**: 669-671.

Wulf, GM, Liou, YC, Ryo, A, W., LS and Lu, KP (2002) Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage. *J Biol Chem* **277**: 47976-47979

Wulf, GM, Ryo, A, Wulf, GG, Lee, SW, Niu, T and Lu, KP (2001) Pin1 is overexpressed in breast cancer and potentiates the transcriptional activity of phosphorylated c-Jun towards the cyclin D1 gene. *EMBO J.* **20**: 3459-3472

Yaffe, MB, Schutkowski, M, Shen, M, Zhou, XZ, Stukenberg, PT, Rahfeld, J, Xu, J, Kuang, J, Kirschner, MW, Fischer, G, Cantley, LC and Lu, KP (1997) Sequence-specific and phosphorylation-dependent proline isomerization: A potential mitotic regulatory mechanism. *Science* **278**: 1957-1960

Yeh, E, Cunningham, M, Arnold, H, Chasse, D, Monteith, T, Ivaldi, G, Hahn, WC, Stukenberg, PT, Shenolikar, S, Uchida, T, Counter, CM, Nevins, JR, Means, AR and Sears, R (2004) A signalling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. *Nat Cell Biol* **6**: 308-318

Yu, Q, Geng, Y and Sicinski, P (2001) Specific protection against breast cancers by cyclin D1 ablation. *Nature* **411**: 1017-1021

Zacchi, P, Gostissa, M, Uchida, T, Salvagno, C, Avolio, A, Voliniak, S, Ronai, Z, Blandino, G, Schneider, C and Del Sal, G (2002) The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. *Nature* **419**: 853-857

Zheng, H, You, H, Zhou, XZ, Murray, SA, Uchida, T, Wulf, G, Gu, L, Tang, X, Lu, KP and Xiao, ZXJ (2002) The prolyl isomerase Pin1 is a regulator of p53 in genotoxic response. *Nature* **419**: 849-853

Zhou, XZ, Kops, O, Werner, A, Lu, PJ, Shen, M, Stoller, G, Küllertz, G, Stark, M, Fischer, G and Lu, KP (2000) Pin1-dependent prolyl isomerization regulates dephosphorylation of Cdc25C and tau proteins. *Mol Cell* **6**: 873-883

Figure Legends

Figure 1. Expression of Pin1 and transgenes in mammary glands normal and cancer tissues derived from the crossbreeding.

(**A-C**) Pin1 protein is absent in Pin1-deficient (Pin1-/-) mice (**A**), but remains at Pin1+/+ levels in Pin1 heterozygote (Pin1+/-) mice (**B**). Mammary glands and breast cancer tissues from littermates with indicated genotypes were homogenized and equal amounts of total protein were separated on SDS-containing gels and transferred to membranes. The membranes were cut into two pieces and subjected to immunoblotting analysis with antibodies against to Pin1 and tubulin (**A, B**), followed by semi-quantified using Imagequant. The Pin1/tubulin ratio was obtained for mammary glands from 4 different animals and presented in (**C**). Note that Pin1 levels in c-Neu or Ha-Ras transgenic mice are variable, but generally higher than in non-transgenic mice (**A, C**). There was no statistically significant difference in Pin1 levels between Pin1+/+ and Pin1+/- mice.

(**D, E**). Pin1 ablation does not affect the expression of the transgenes Ha-Ras or c-Neu.

Protein lysates or tissue sections of mammary glands of the specified genotypes were subjected to immunoblotting (**D**) or immunostained (**E**) with anti-c-Neu or anti-Ha-Ras antibodies. Note that out of 3-5 mice analyzed each group, there was no statistically significant difference in Neu or Ras levels between Pin1+/+ and Pin1-/- mice.

Figure 2. Pin1 ablation is highly effective in preventing breast cancers induced by MMTV-Neu or -Ras, but not -Myc

Transgenic mice overexpressing activated c-Neu, Ras or Myc under the control of the promoter MMTV were crossbred with Pin1-/- mice to generate mice with nine different genotypes. Virgin females were observed for 75 weeks. Breast cancers were recorded at the time of first palpation.

Figure 3. Pin1 ablation effectively blocks the induction of cyclin D1 by Neu or Ras

Protein lysates or tissue sections of mammary glands from virgin littermates of the specified genotypes were subjected to immunoprecipitation with anti-cyclin D1 or control IgG, followed by

immunoblotting with anti-cyclin D1 antibodies (**A**) or to immunohistochemistry with anti cyclin D1 antibodies (**B**). Note that similar results were obtained in at least 4-5 mice each group examined.

Figure 4. Pin1 ablation does not affect the differentiation of primary MECs in 3D cultures.
Primary MECs were isolated from morphologically and histologically normal mammary glands of non-transgenic (**A**) or Neu transgenic (**B**) littermates in Pin1+/+ or Pin1-/- background at ages of 3-4 months. After culture in collagen-coated plates for 3-5 days, MECs were plated as single cell suspension in reconstituted basement membrane (Matrigel) and analyzed at the indicated time points. Phase images were taken on 5, 10 and 20 days in culture, followed by fixation and confocal immunofluorescence staining with anti-E-cadherin antibodies.

Figure 5. Characterization of abnormal differentiation patterns of MECs derived from Neu or Ras transgenic mice in Pin1+/+ or Pin1-/- genetic background.
Primary MECs were isolated from littermates with different genetic background and subjected to 3D cultures in reconstituted basement membrane for 20 days. Colonies were analyzed by phase contrast microcopy to reveal the morphology (**A, F**), fixed and stained with hematoxylin and eosin to reveal the histology (**B, G**), stained with anti-E-cadherin antibodies to reveal the cell polarity (**C, H**), with anti-α6 integrin to reveal the base membrane integrity (**D**), with anti-Ki67 antibodies to reveal cell proliferation (**E**). Based on these assays, colonies are divided into three categories, namely "Regular", "Irregular" and "Cancer-like". Arrows in (**A, F**) point to cell surface spikes protruding into the Matrigel, while an arrows in (**B**) points to a dividing cell. (**A, B, F, G**) Light microscopy at 200x; (**C-E, H**), confocal fluorescence microscopy at 200x.

Figure 6. Non-neoplastic primary MECs of Neu or Ras mice in the Pin1+/+, but not Pin1-/- background exhibit the malignant phenotype, including forming tumors in nude mice.
(**A-D**) Primary MECs were isolated from littermates with different genetic background and subjected to 3D cultures in reconstituted basement membrane for 20 days. Assays were set up for 3

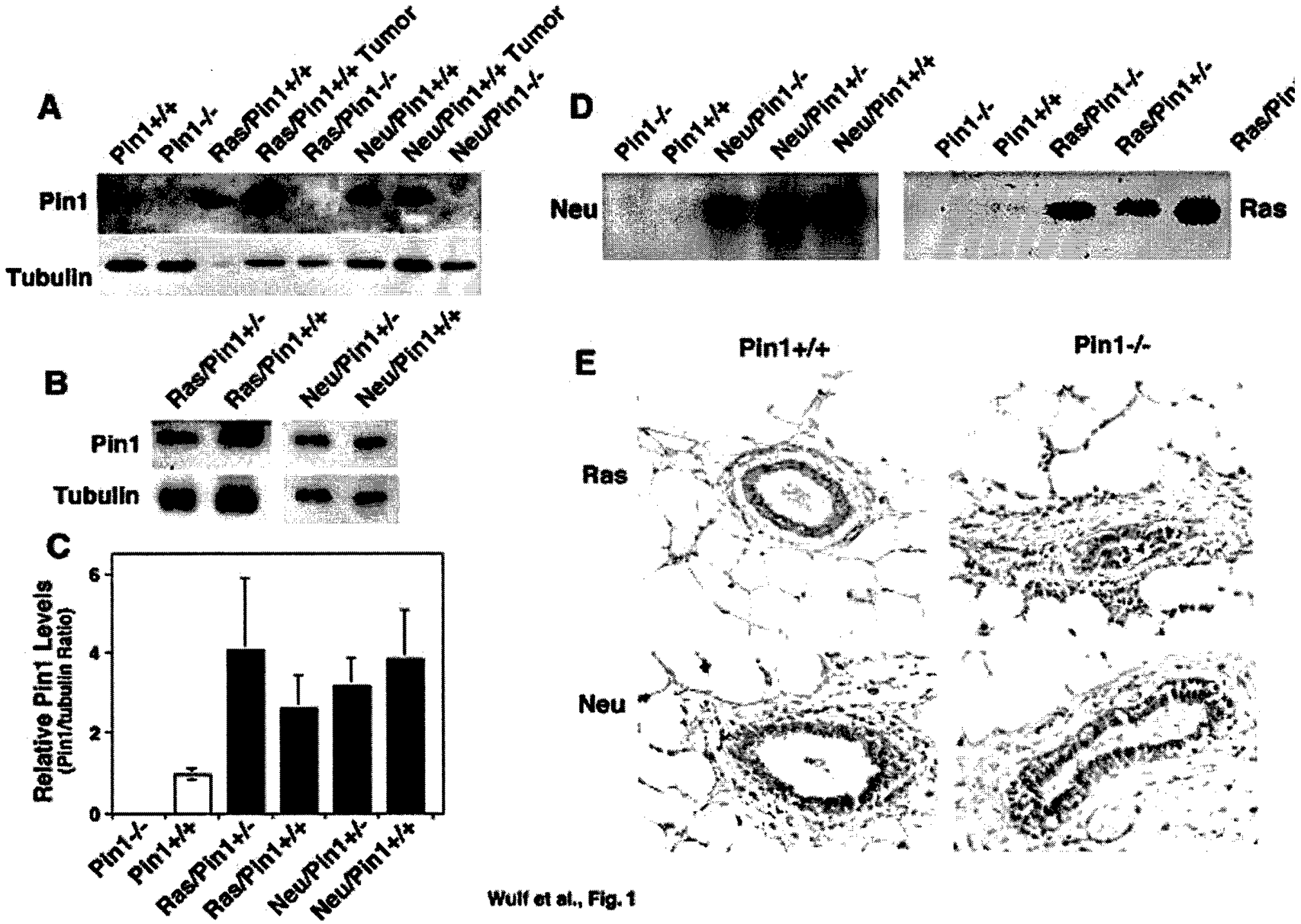
to 5 mice of each genotype, plated in quadruples. Colonies were categorized and counted under phase microscopy. The number of colonies in different categories per 10,000 cells plated was plotted as mean ± SD, with p values being indicated. N.S., no significant.

(**E**) Secondary colony formation. "Regular" and "Irregular" colonies derived from Neu or Ras MECs in Pin1+/+ or Pin1-/- background in 3D cultures were picked separately at 21 days and trypsinized, followed by a more round of 3D cultures for 20 days.

(**F**) MEC colonies derived from Neu transgenic mice only in Pin1+/+, but not Pin1-/- background give rise to tumors in nude mice. Day 21 colonies were harvested and resuspended in 100 μl MEGM/4% Matrigel, followed by injecting subcutaneously into female nude mice in duplicates each (right and left flank). Out of 6 injections of three mice each group, three tumors were derived from Neu/Pin1+/+ colonies, but Neu/Pin1-/- colonies did not generate any tumors.

Figure 7. Expression of cyclin D1 or its T286A mutant restores the malignant phenotype of Neu/Pin1-/- primary MECs.

Primary MECs derived from Neu/Pin1-/- mice were infected with retroviruses for either control, cyclin D1 or cyclin $D1^{T286A}$, followed by 3D culture on Matrigel. Expression of cyclin D1 in infected MECs was monitored by Western Blotting (Fig. S4). At day 21, colonies were analyzed by phase contrast microcopy to reveal the morphology (**A**), fixed and stained with anti-α6 integrin antibodies to assay basement membrane integrity (**B**).



Wulf et al., Fig. 1

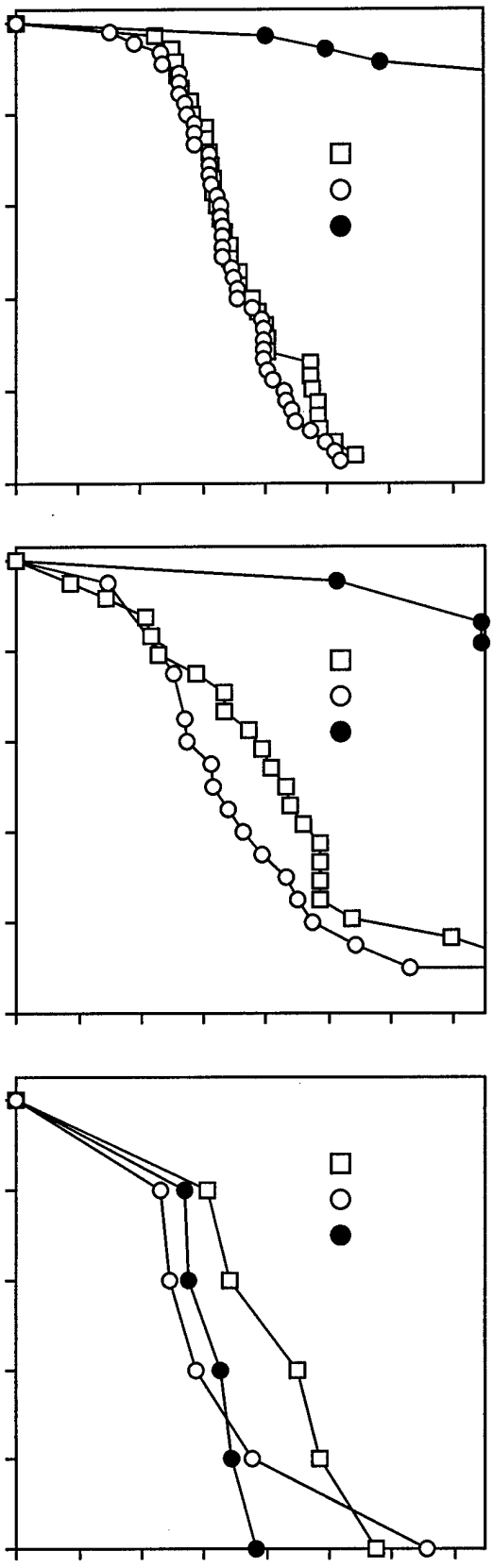
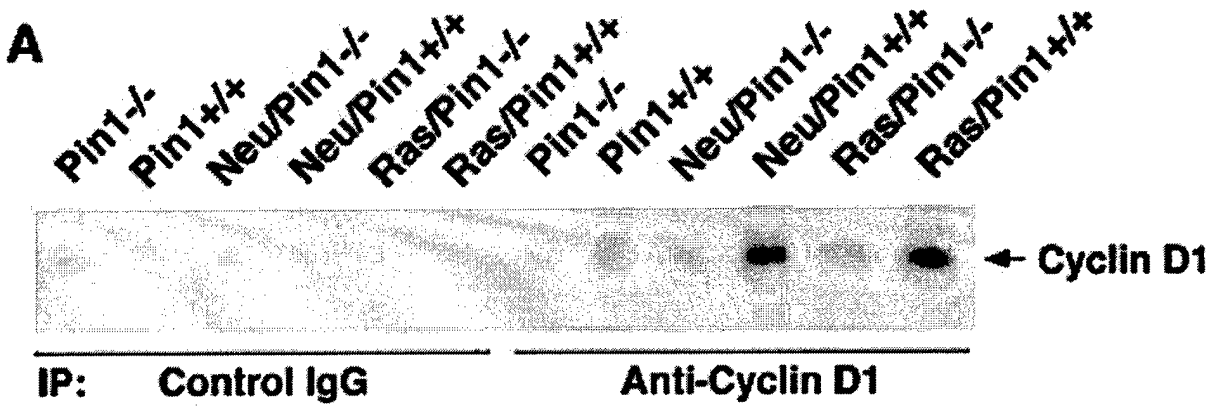
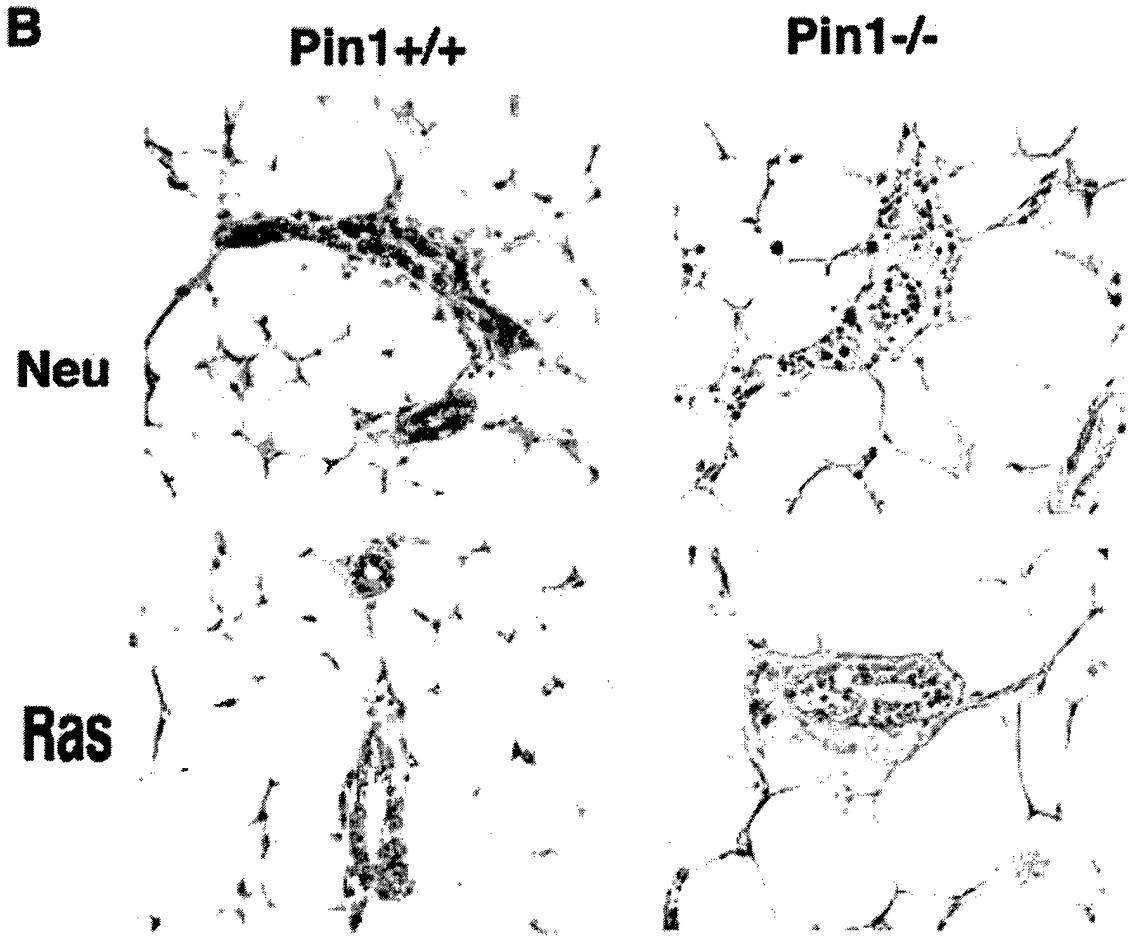
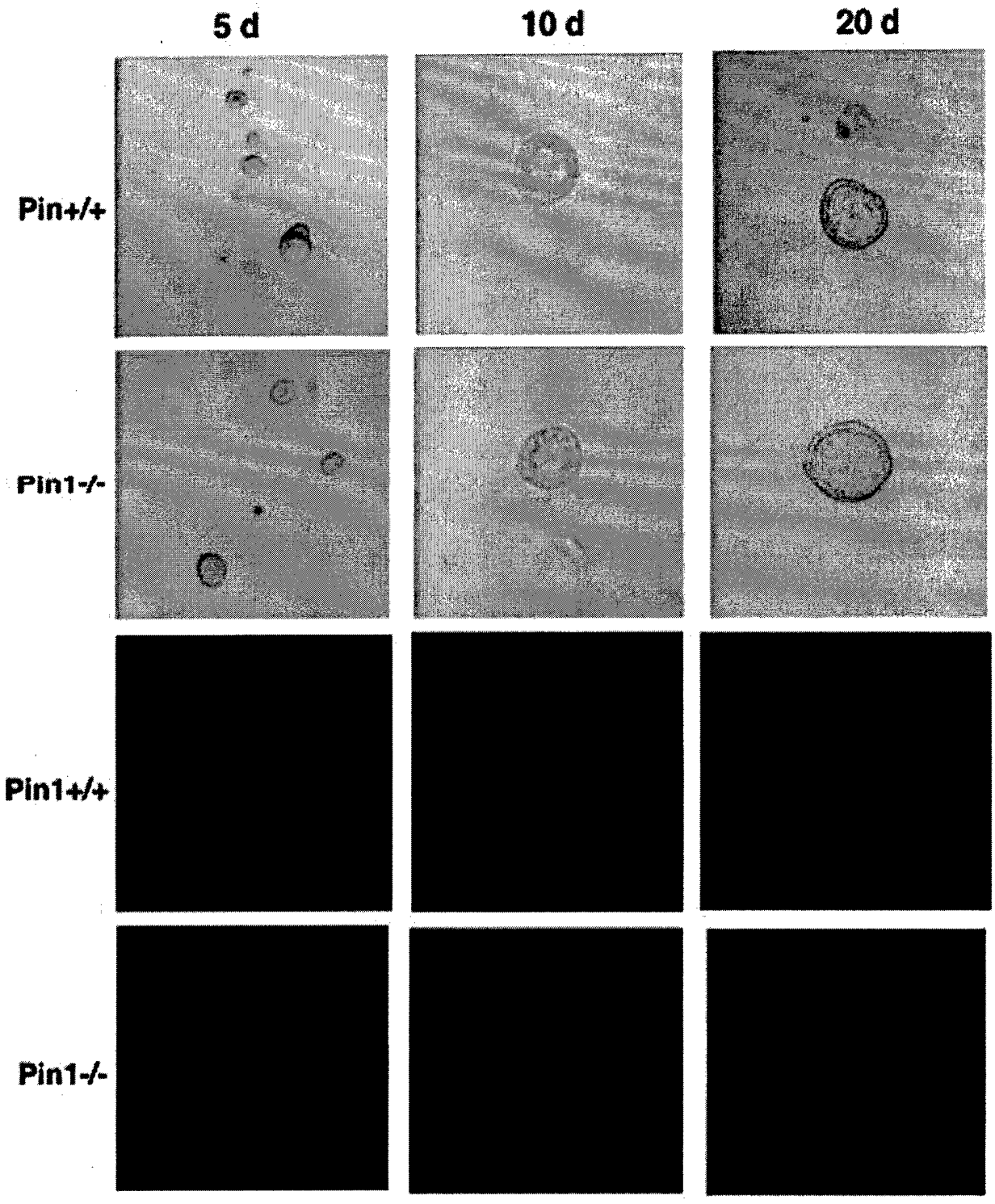


Figure 2

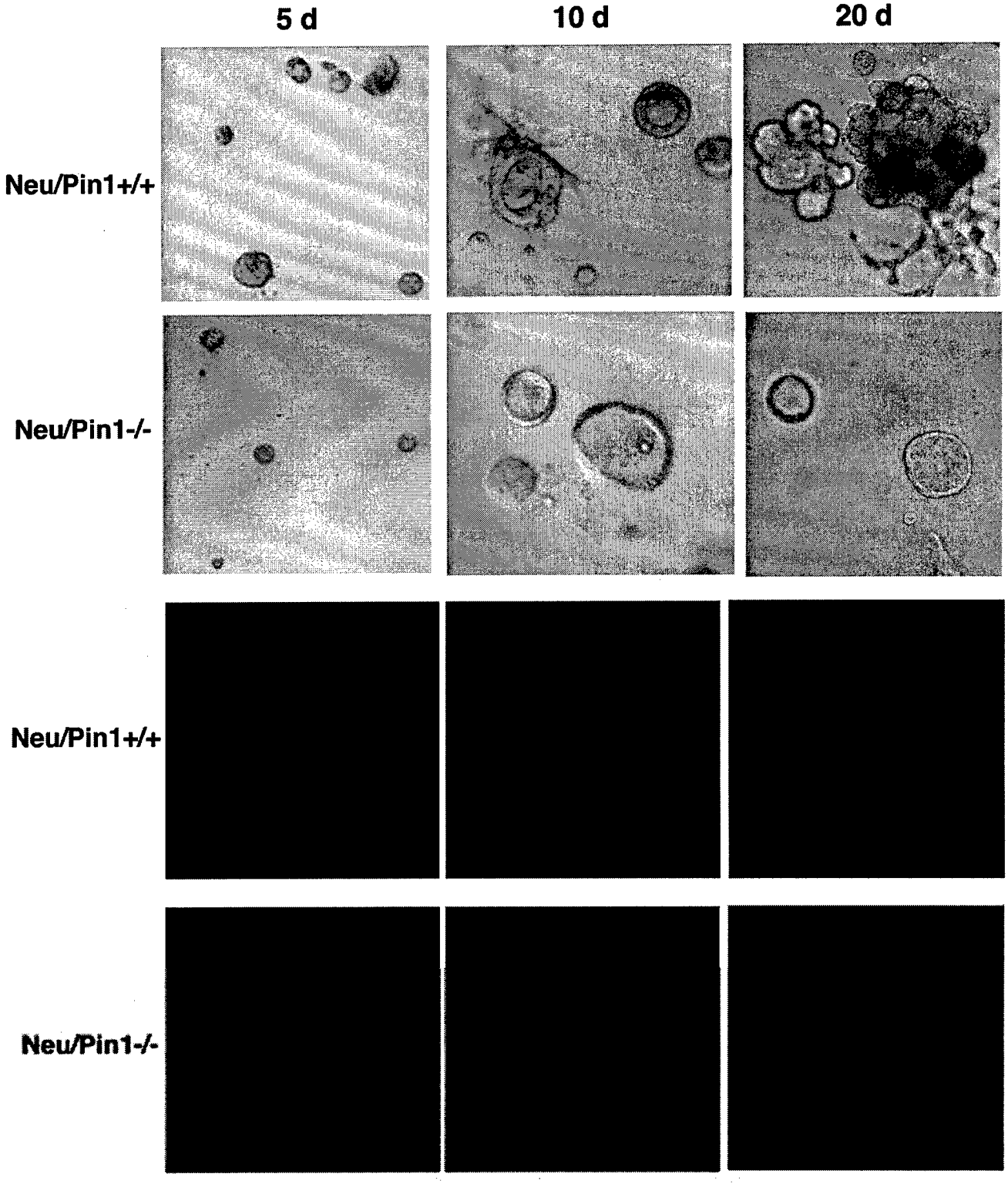




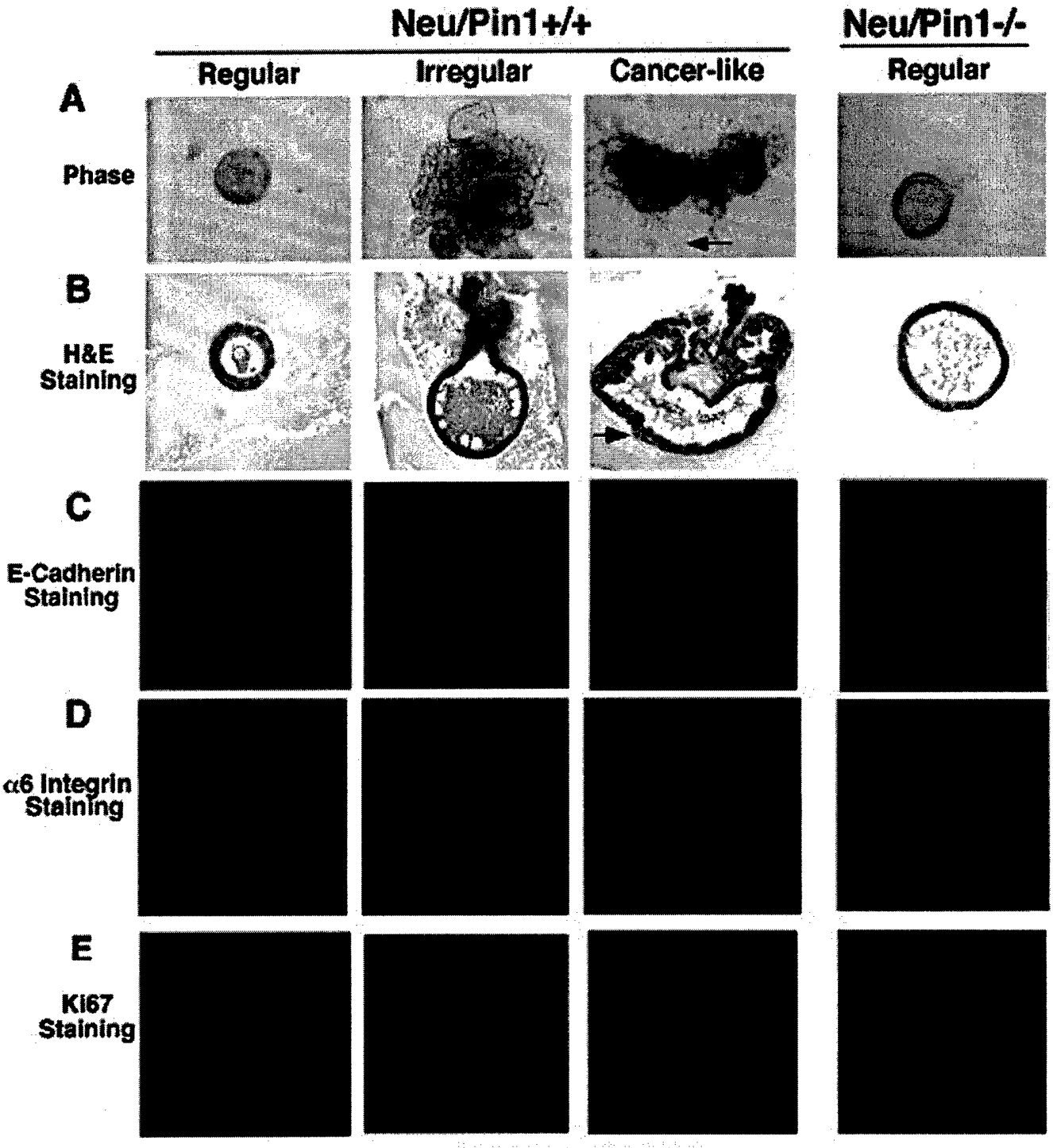
Wulf et a., Fig. 3



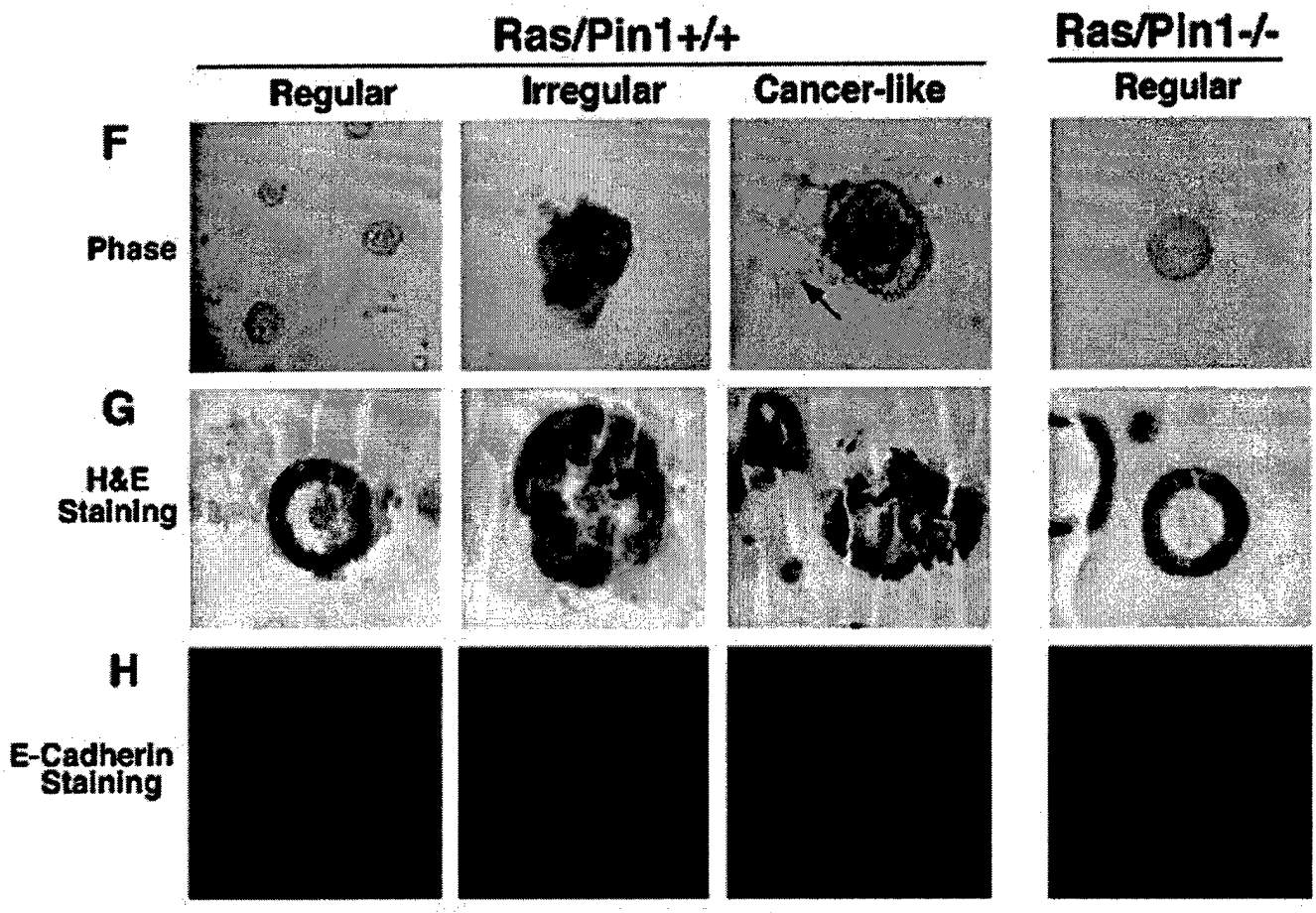
Wulf et al., Fig. 4A



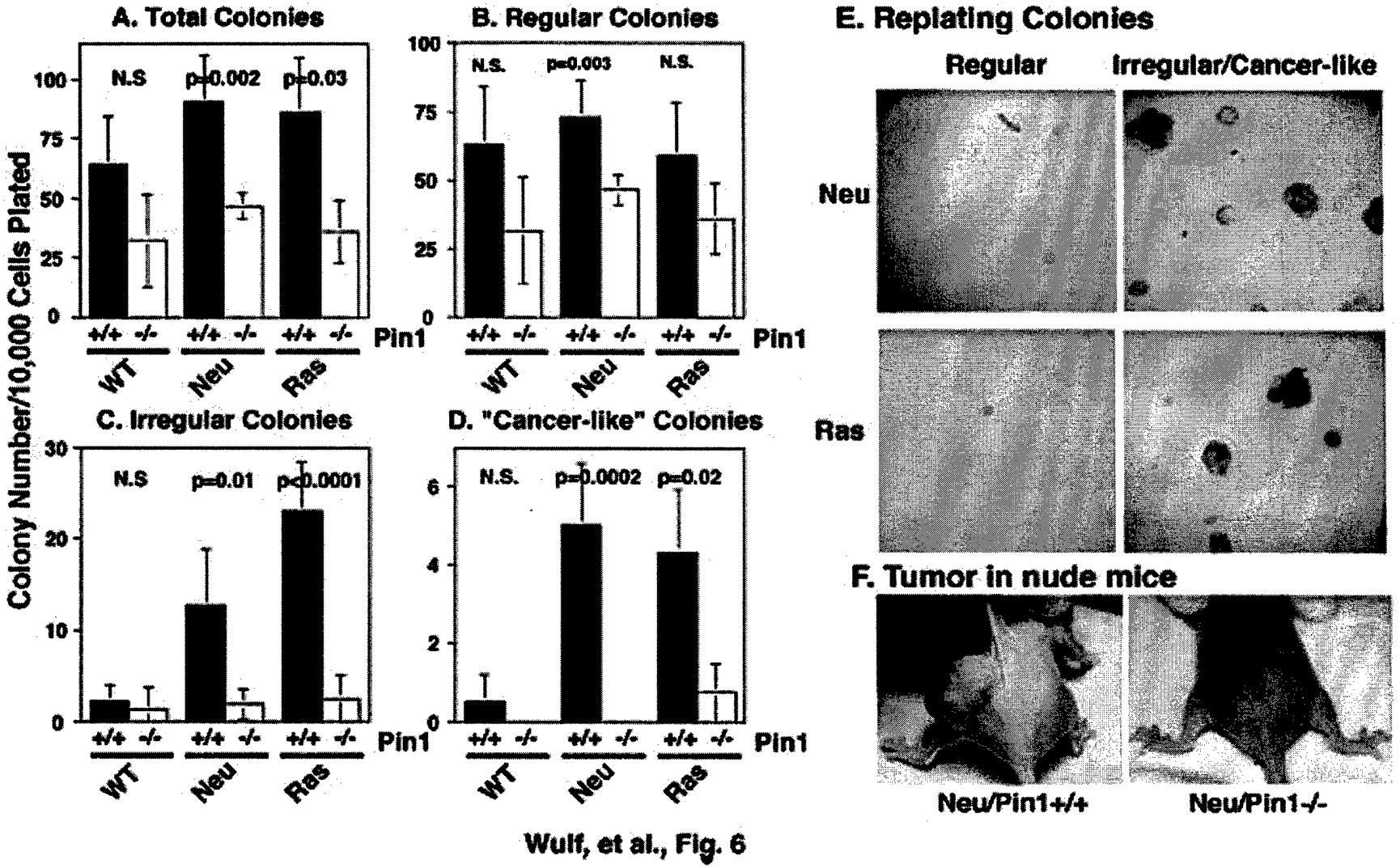
Wulf et al., Fig. 4B



Wulf et al., Fig. 5A-E

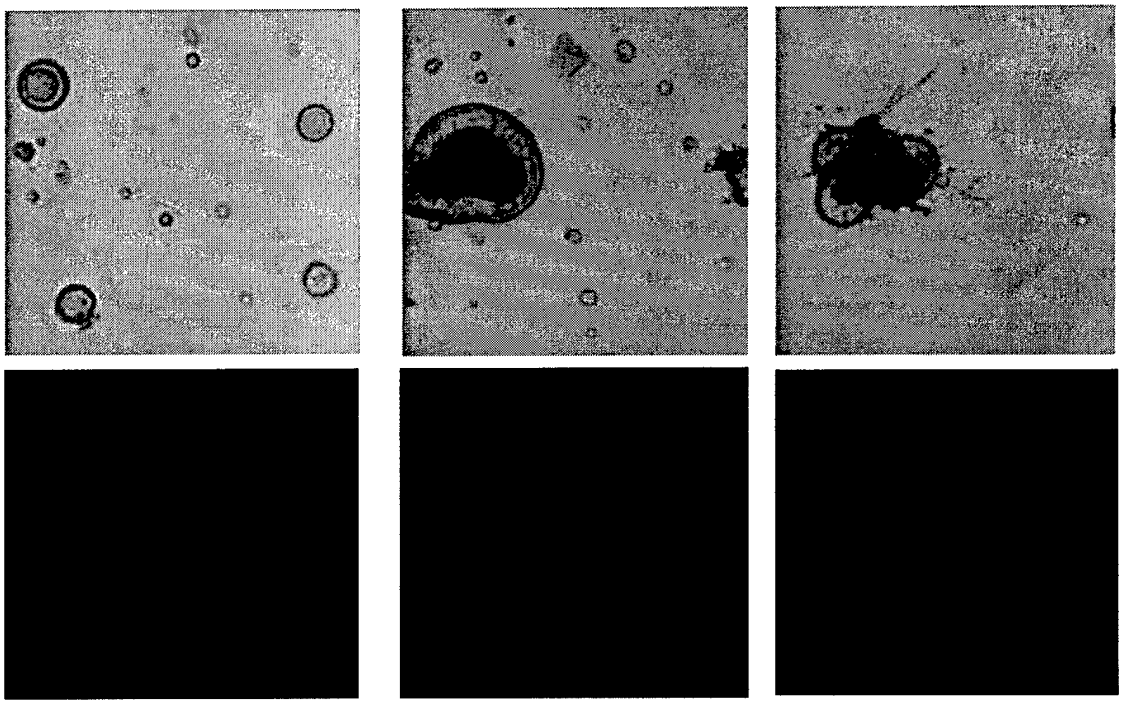


Wulf et al., Fig. 5F-H



Wulf, et al., Fig. 6

A



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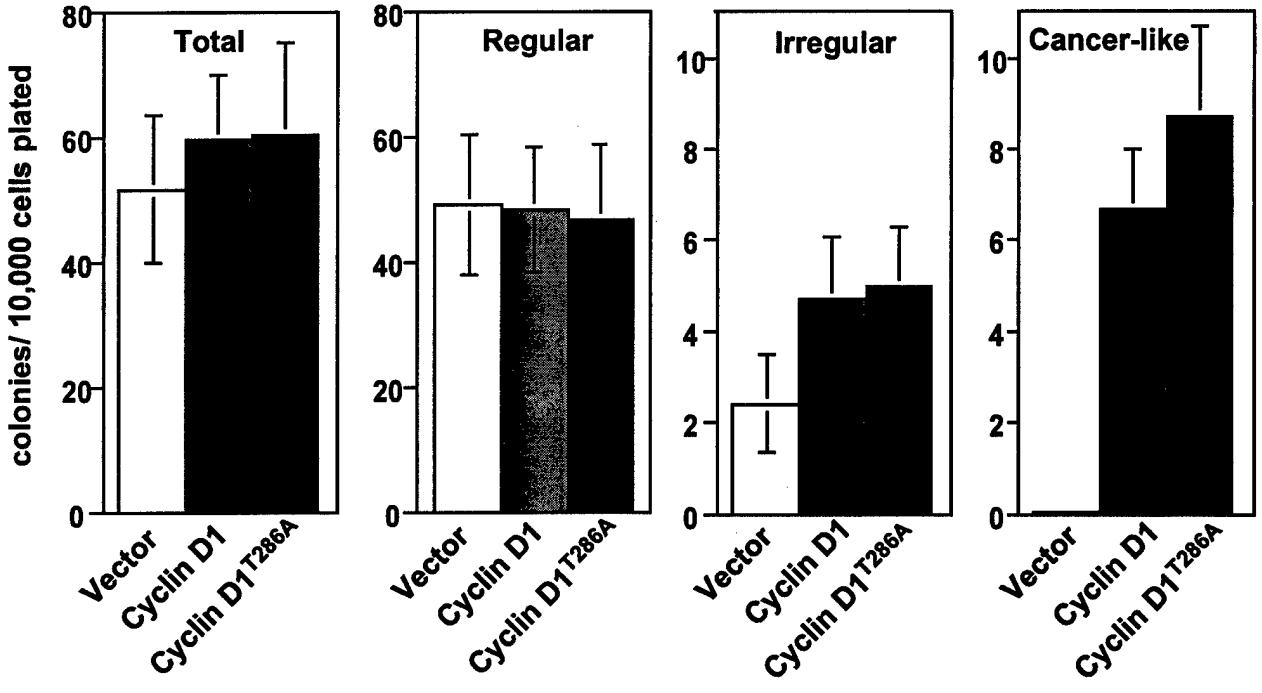
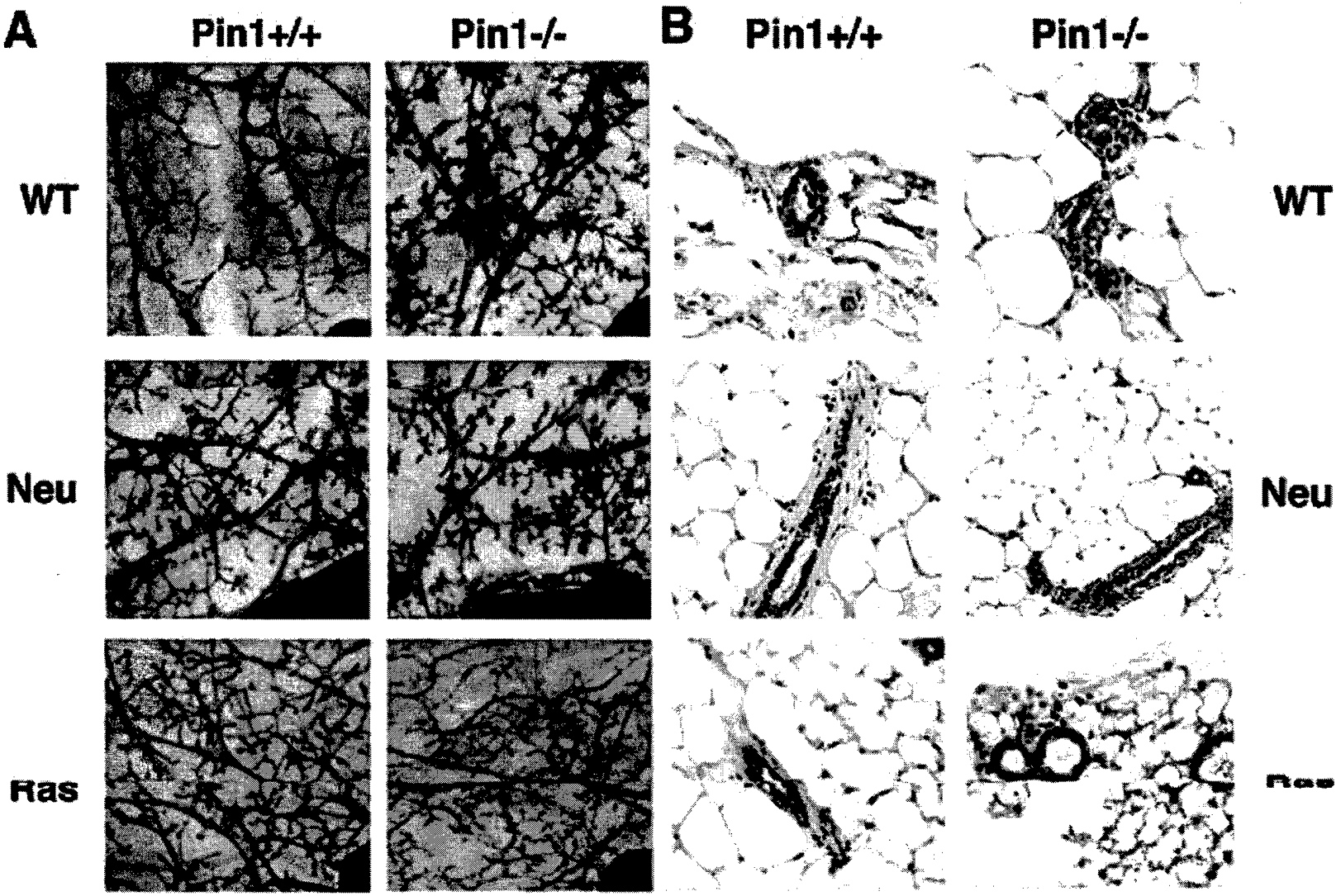

colonies/ 10,000 cells plated
Total
Regular
Irregular
Cancer-like
0
20
40
60
80
2
4
6
8
10
Vector
Cyclin D1
Cyclin D1T286A

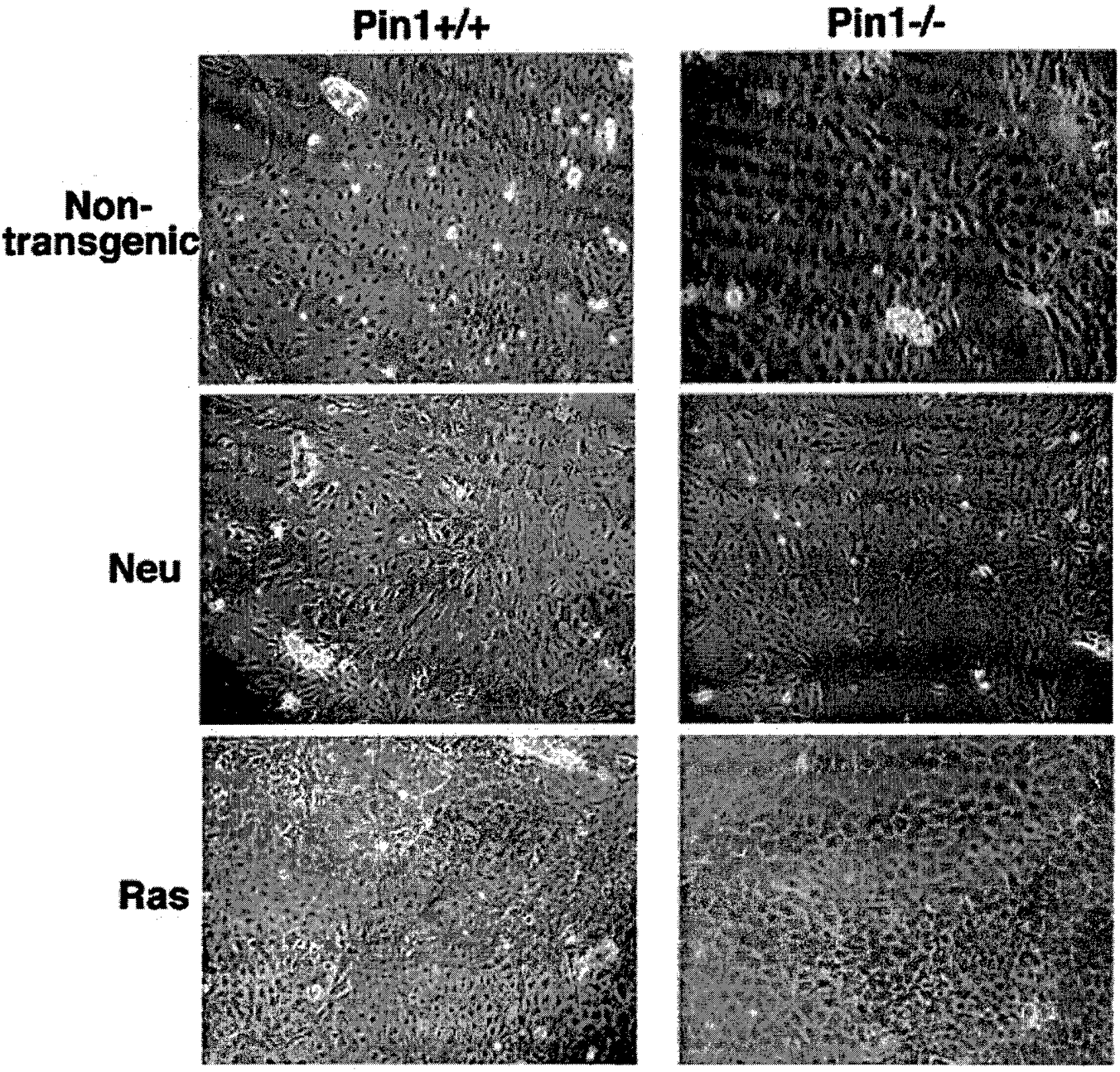
Figure 7



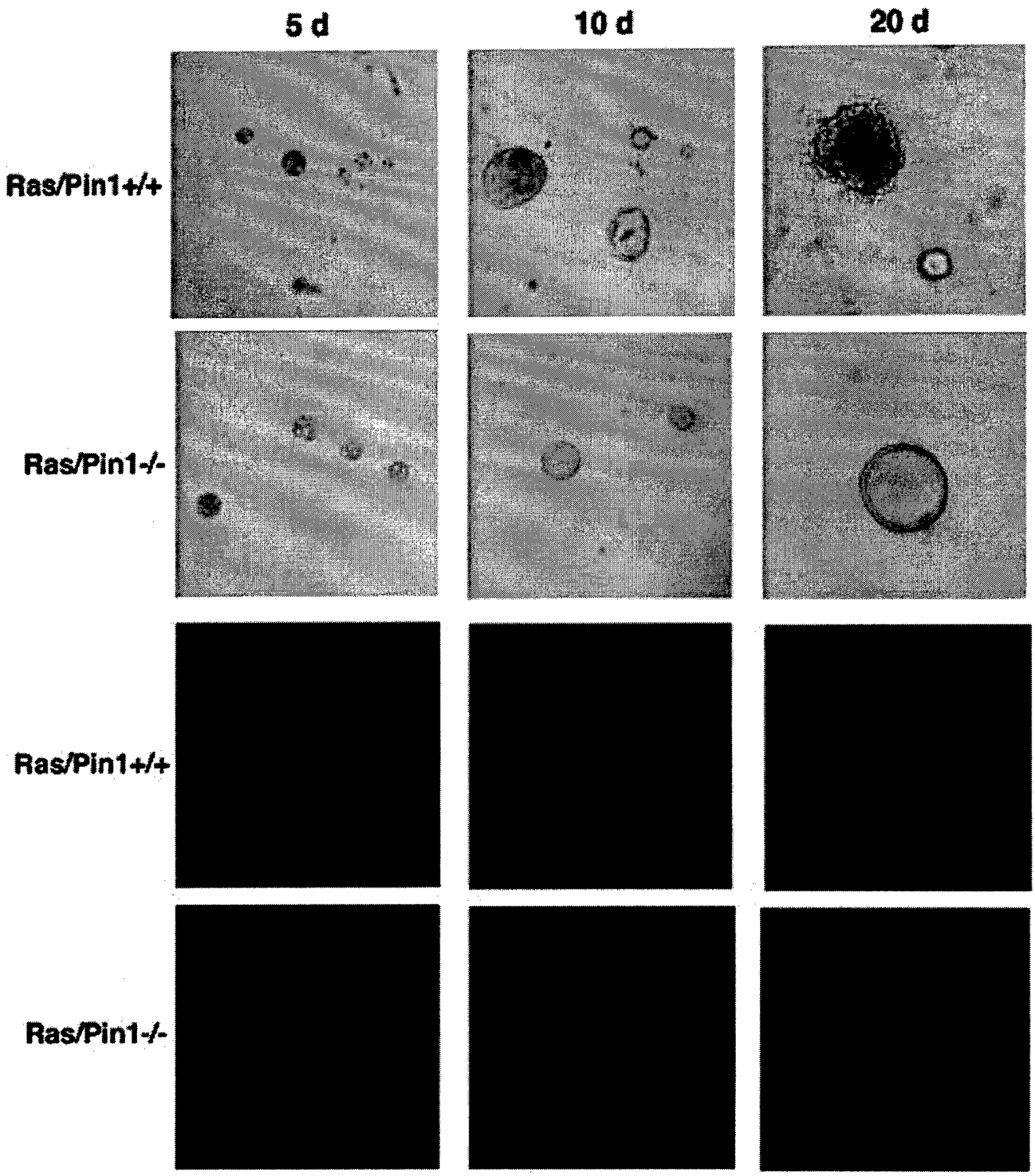
Wulf et al., Fig. S1

Supplemental Fig. S1. Pin1 ablation does not affect the development of virgin mammary glands.

The whole mounts of inguinal mammary glands were prepared and the epithelial component was stained with carmine red (A). Histological sections were stained with hematoxylins and eosin (B). Morphology of the mammary glands obtained from virgin mice reveals no differences between the various genotypes.

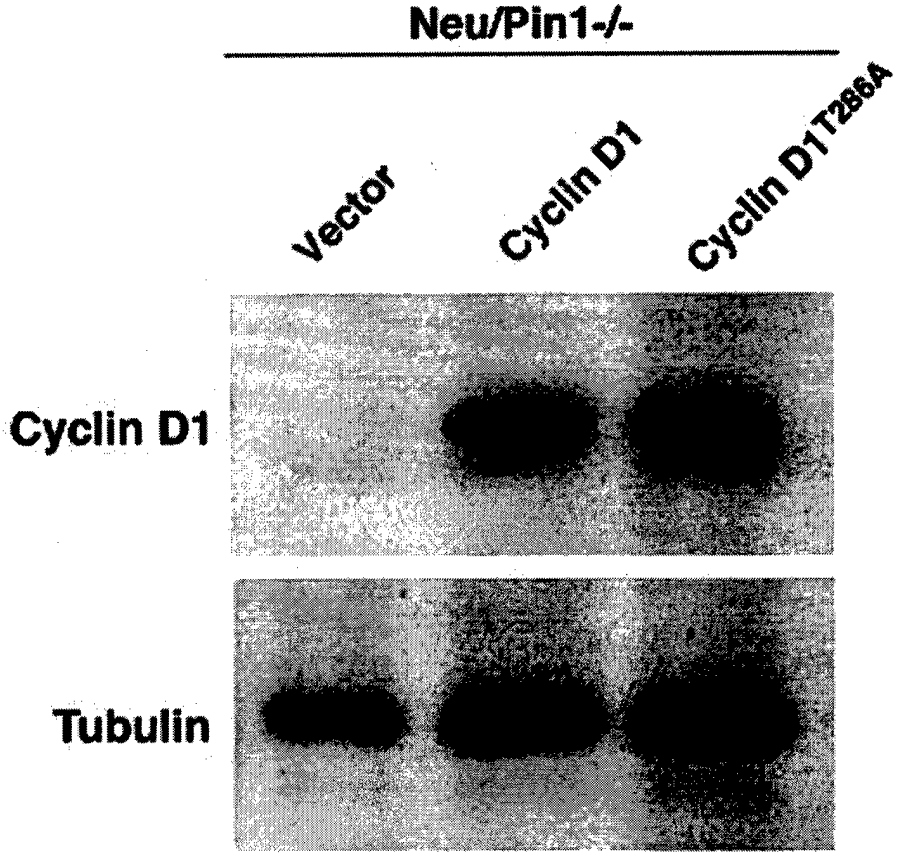


Supplemental Figure S 2 Primary MECs derived from transgenic and non-transgenic mice inPin1 +/+ and Pin1-/- genetic background display similar growth patterns in 2D culture.



Wulf., et al., Fig. S3

Supplemental Figure S3. Differentiation time course of primary MECs derived from Ras transgenic mice in Pin1+/+ or Pin1-/- genetic background in 3-D cultures.



Wulf et al., S4

Supplemental Fig. S4. Retroviral infection of primary MECs derived from MMTV-Neu/Pin1-/- mice with control vector virus or virus expressing cyclin D1 or it mutant, followed by immunoblotting analysis with anti-cyclin D1 or tubulin antibodies.